BIOTECHNOLOGICAL APPLICATION OF STEROIDOGENIC CYTOCHROMES P450

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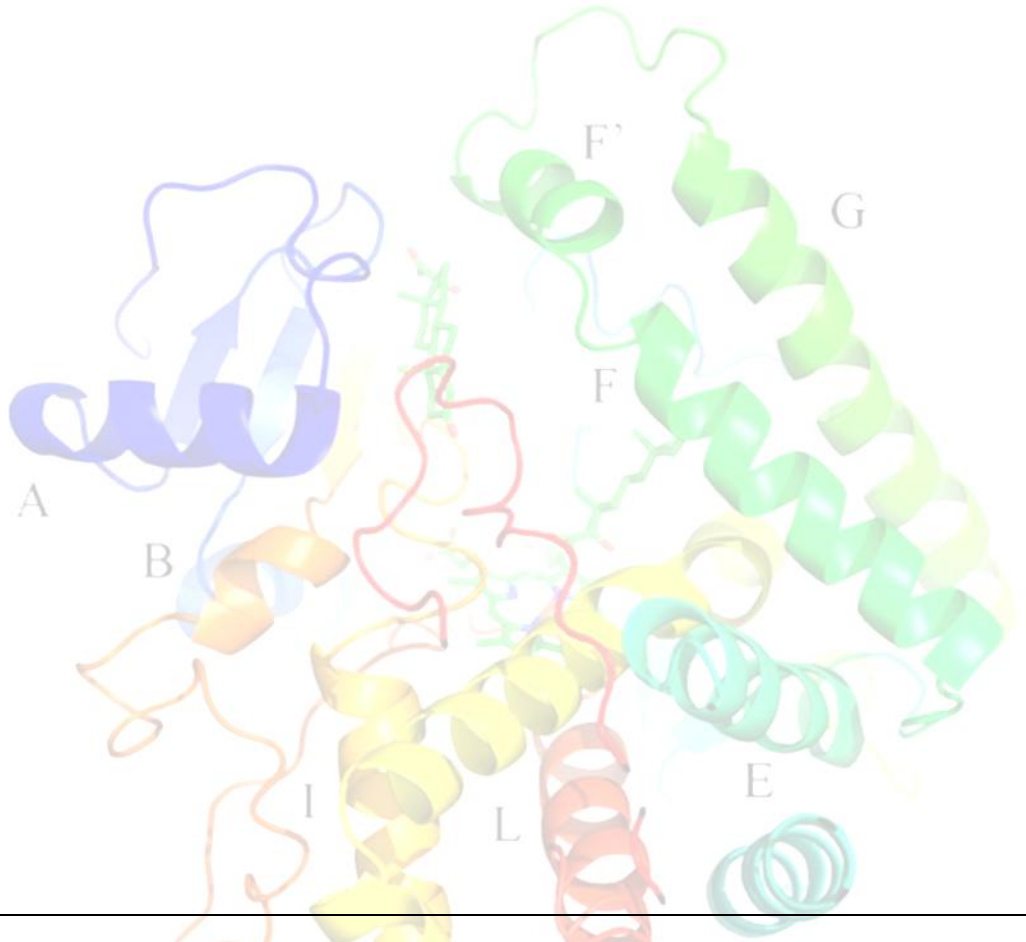

Tag des Kolloquiums:	**20.05.2016**
Dekan:	**Prof. Dr. Dirk Bähre**
Berichterstatter:	**Prof. Dr. Rita Bernhardt**
	Prof. Dr. Gert-Wieland Kohring
Vorsitz:	**Prof. Dr. Uli Müller**
Akad. Mitarbeiter:	**Dr. Judith Becker**

Water mark from [102].

After you reach a certain level of talent (and quite a few have that talent) the deciding factor is ambition, or as I see it, how much you really need. Need to be loved and need to be proud of yourself. And I guess that's what ambition is - it's not all a depraved quest for position… or money.
Maybe it's for love.

Janis Joplin
(1943 – 1970)

Danksagung

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Scientific contributions

This work is based on five original research papers reproduced in **Chapter 2**. The original manuscripts are printed with the kind permissions from BioMedCentral, *Microbial Cell Factories* (2.1 Brixius-Anderko et al. (2015); 2.3 Schiffer et al. (2015a)), Wiley, *Biotechnology and Applied Biochemistry* (2.2 Brixius-Anderko et al. (2016)), ASPET, *Drug Metabolism and Disposition* (2.4 Schiffer et al. (2016)) and Elsevier, *The Journal of Steroid Biochemistry and Molecular Biology* (2.5 Schiffer et al. (2015b)).

2.1 **Simone Brixius-Anderko,** Lina Schiffer, Frank Hannemann, Bernd Janocha, Rita Bernhardt, „A CYP21A2 based whole-cell system in *Escherichia coli* for the biotechnological production of Premedrol", *Microbial Cell Factories*, 2015, 14:135 doi:10.1186/s12934-015-0333-2

The author carried out the described *in vitro* experiments with purified enzymes, designed the vectors for whole-cell bioconversion and established the here described CYP21A2-based whole-cell systems. Furthermore, the author performed all experiments in shaking flasks and contributed to writing the manuscript.

Patent with Sanofi Aventis, "21-Hydroxylation of Steroids"; Submission number 1000263078; Application number EP14306740.3; Submission: 30 October 2014

2.2 **Simone Brixius-Anderko**, Frank Hannemann, Michael Ringle, Yogan Khatri, Rita Bernhardt. (2016) "An indole deficient *Escherichia coli* strain improves screening of cytochromes P450 for biotechnological applications", *Biotechnology and Applied Biochemistry*, accepted 18-02-2016.

The author produced all described *in vitro* data including dissociation constants with purified enzymes, created the strain C43(DE3)_Δ*tnaA* and carried out the whole-cell biotransformation experiments. The author also contributed to writing the manuscript.

2.3 Lina Schiffer, **Simone Anderko,** Anna Hobler, Frank Hannemann, Norio Kagawa, Rita Bernhardt, "A recombinant CYP11B1 dependent *Escherichia coli* biocatalyst for selective cortisol production and optimization towards a preparative scale", *Microbial Cell Factories*, published online 2015 Feb 25. doi: 10.1186/s12934-015-0209-5

The author established the CYP11B1-dependent whole-cell system as well as the fluorescence based screening system for molecular evolution of CYP11B1.

2.4 Lina Schiffer, **Simone Brixius-Anderko**, Frank Hannemann, Josef Zapp, Jens Neunzig, Mario Thevis, Rita Bernhardt, "Metabolism of oral-turinabol by human steroid hormone-synthesizing cytochromes P450", *Drug Metabolism and Disposition*, 2016 Feb;44(2):227-37. doi: 10.1124/dmd.115.066829

The author established the CYP11B1- and CYP11B2-based whole-cell systems, which were used to produce the oral-turinabol metabolites.

2.5 Lina Schiffer, **Simone Anderko**, Frank Hannemann, Antje Eiden-Plach, Rita Bernhardt, "The CYP11B subfamily", *The Journal of Steroid Biochemistry and Molecular Biology*, available online 18 October 2014, doi:10.1016/j.jsbmb.2014.10.011.

The author contributed to literature research and to writing the manuscript.

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Abstract

Synthetic glucocorticoids are of high pharmaceutical value due to their anti-inflammatory and immunosuppressive effects. The low-yield chemical synthesis of glucocorticoids consists of multiple steps and includes toxic compounds as well as unwished by-product formation. Thus, one intends to replace this process by a more sustainable biocatalytic alternative.

The work presented here demonstrates an enzyme-mediated approach to produce the synthetic glucocorticoid premedrol, a precursor of methylprednisolone (medrol), by a one-step hydroxylation at carbon atom 21catalyzed by bovine CYP21A2, which belongs to the protein superfamily of cytochromes P450. Therefore, a CYP21A2-based whole-cell system was established, by which a maximum yield of 0.65 g/L premedrol could be achieved - a promising perspective for an industrial application.

With regard to a molecular evolution of CYP21A2 as well as an elucidation of the biocatalytic ability of orphan CYPs in a high-throughput microtiter scale, the indole-deficient *Escherichia coli* strain C43(DE3)_Δ*tnaA* was generated, since indole is an inhibitor of some CYPs and massively impedes a screening. Applying C43(DE3)_Δ*tnaA* in biotransformation resulted in a significantly higher product formation in case of CYP21A2 as well as of CYP264A1 from the myxobacterium *Sorangium cellulosum* So ce56 and thus, serves as convenient host in future screening procedures.

Zusammenfassung

Synthetische Glucocorticoide sind aufgrund ihrer antiinflammatorischen und immunsuppressiven Wirkung von hohem pharmazeutischem Wert. Die chemische Synthese von Glucocorticoiden besteht aus mehreren Schritten und beinhaltet die Verwendung toxischer Substanzen als auch die Bildung unerwünschter Nebenprodukte. Daher soll dieser Prozess durch ein nachhaltigeres biokatalytisches Verfahren ersetzt werden.

Die vorliegende Arbeit demonstriert eine biokatalytische Herangehensweise zur Produktion des synthetischen Glucocorticoids Premedrol, einem Vorläufer von Methylprednisolone (Medrol), durch eine einstufige Hydroxylierung am C21, welche durch das zur Proteinsuperfamilie der Cytochrome P450 gehörende steroidogene bovine CYP21A2 katalysiert wird. Daher wurde ein CYP21A2-basiertes Ganzzellsystem etabliert, mit Hilfe dessen eine maximale Premedrolausbeute von 0,65 g/l erzielt werden konnte – eine vielversprechende Perspektive im Hinblick auf eine industrielle Anwendung.

Hinsichtlich einer molekularen Evolution von CYP21A2 und einer Aufklärung des biokatalytischen Potentials unbekannter CYPs durch ein Hochdurchsatz-Screening wurde der Indol defiziente *Escherichia coli* Stamm C43(DE3)_Δ*tnaA* generiert, da Indol einige CYPs inhibiert und somit ein Screening erschwert. Ein Umsatz mit C43(DE3)_Δ*tnaA* resultierte in einer signifikant höheren Produktbildung im Falle des CYP21A2 und des CYP264A1 aus *Sorangium cellulosum* So ce56 und ist somit ein geeigneter Wirtsorganismus für künftige Screeningverfahren.

1. Introduction

1.1 Biocatalysis of today

"All our efforts to defeat poverty and pursue sustainable development will be in vain if environmental degradation and natural resource depletion continue unabated."

Kofi Annan

With respect to global warming, the increase of greenhouse gas emissions as well as the overexploitation of fossil fuels, our civilized culture has to pursue sustainable alternatives to meet the needs of our society in the future. This includes not only a switch to renewable energy sources, but also a revolution in terms of the industrial production of bulk and fine chemicals, pharmaceuticals and food additives. Commonly used chemical synthesis often consists of multistep-reactions and needs harsh reaction conditions as well as the use of polluting substances like heavy metals and halogens. Hence, one has to promote sustainable, environmentally friendly alternatives by employing nature´s repertoire of naturally occurring enzymatic reactions [1]. In contrast to chemical synthesis enzymes perform highly specific reactions, ensuring enantio-, regio- and stereoselectivity, which reduces unwished by-products to a minimum and avoids the need of protection groups. Furthermore, they work under mild reactions conditions without the need of environment-polluting, toxic compounds. While biocatalysis in general was subjected to some limitations in former decades, like poor protein stability and low turnover numbers, biocatalysis of today benefits from modern protein engineering techniques. The broad field of enzyme improvement unites bioinformatics, biology, chemistry, engineering and biotechnology to an interdisciplinary research for an effective application of enzyme-mediated reactions. There exist several approaches to find and to improve an enzyme of interest, performing a particular reaction. Apart from the classical screening of microorganisms, the molecular evolution of proteins by directed and undirected mutagenesis is a convenient method to improve the stability and the catalytic activity [2]. Furthermore, enzyme mutagenesis can lead to changed stereo- and enantioselectivity, designing the reaction according to the requirements [3, 4]. Application of bioinformatics is an important achievement within modern biocatalysis, since *in silico* modeling of enzymes help to predict the impact of changes in the active site concerning selectivity and

substrate binding [5-7]. This powerful tool helped in former years to apply improved enzymes for a biocatalytic process, which takes either place with purified proteins in one-pot reactions or enzyme cascades under defined *in vitro* conditions or within the scope of metabolic engineering and synthetic biology *in vivo* [8-10]. The latter ones deal with the heterologous protein expression in a suitable microbial host as well as the modification of the host´s metabolic pathways for the production of a particular compound. The achievements in biocatalysis led to industrial application of some processes in recent years. The chemical synthesis of acrylamide, the precursor of polyacrylamide, which is used in wastewater treatment, petroleum recovery or paper making, involves the polluting catalysts copper and sulfuric acid and is performed under harsh reaction conditions. The biocatalytic alternative uses an overexpression of nitrile hydratase in *Rhodococcus rhodochrous J1* and produces annually over 650,000 tons in Japan under mild and sustainable conditions [11, 12]. The biotechnological production of the hormone insulin, the leading drug in the treatment of diabetics, is done by microbial cell factories, either in *Escherichia coli* or in *Saccharomyces cerevisiae*, and is one of the most impressive achievements of biocatalysis during the last decades, since it ensures a sufficient supply, facing the growing demand [13]. The next section will treat the fascinating and versatile protein superfamily of cytochromes P450 (CYP, P450) whose biocatalytic impact was underestimated for a long time, though their spectrum of catalyzed reactions is highly diverse. In the following, the role of cytochromes P450 in the development of terrestrial life and their high potential as versatile biocatalysts is enlightened.

1.2 Cytochromes P450 in brief

In biocatalysis of today, the protein superfamily of cytochromes P450 cannot be neglected. This group of enzymes is highly conserved through all domains of life and has a strong impact on the development on life on earth. In the 1960s, Omura and Sato observed unique spectral properties of isolated liver microsomes after reduction with sodium dithionate and treatment with carbon monoxide, showing an untypical absorption maximum at 450 nm, and postulated a CO-binding pigment, a heme-protein “with unusual properties” [14]. This study underlined the observation previously reported by Klingenberg et al. [15].

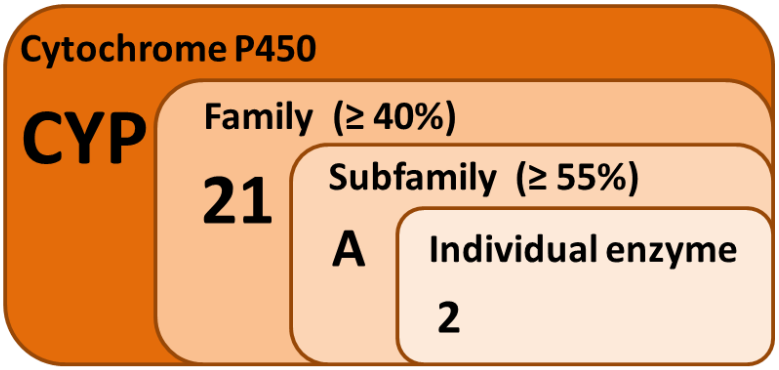

Figure 1: **Schematic illustration of cytochrome P450 classification**. Cytochromes P450 belong to the same CYP family at a sequence similarity of more than 40% (Arabic numbers) and to the same subfamily at a similarity of more than 55% (Arabic letters). The respective individual enzyme is designated with Arabic numbers.

In the following years, the impact of Omura and Sato´s finding became clearer, since essential functions in the human body, like steroid hormone biosynthesis and detoxification in the liver, could be attributed to 57 distinct P450s [16]. With growing progress in molecular biology, more and more CYPs could be isolated from distinct organisms like fungi, bacteria, mammals and plants showing the abundance of CYPs in all domains of life [17]. The role of cytochromes P450 within the evolution of higher forms was elucidated, since they are involved in the synthesis of membrane lipids like ergosterol in fungi and of important steroid hormones, without which metabolic signaling within bigger organisms would be impossible [18, 19]. Apart from their impact on steroid biogenesis, P450s play a crucial role for detoxification processes in mammals, which take place in the liver and include drug and xenobiotics metabolism [16]. Within the phylogenetic branch of bacteria, the role of cytochromes P450 has not been fully understood. In the myxobacterium *Sorangium cellulosum* So ce56, some P450s are clustered with a terpene cyclase and are able to hydroxylate terpenoid substrates [20-22]. Consequently, these enzymes are mainly involved in the synthesis of secondary metabolites, which is underlined by the aspect that cytochromes P450 are also embedded in the biosynthesis of antibiotics in species of the fungus *Streptomyces* [23-25]. Though CYPs share a high similarity within their secondary structure within all species, the primary amino acid sequence is highly diverse among them and even within species [26, 27]. Aside from this exciting diversity, all cytochromes P450

possess a highly conserved cysteinate residue, which is coordinating the prosthetic heme group with a central iron ion, embedded in a protoporphyrin, while other cytochromes like hemoglobin possess a coordinating histidine residue [28].

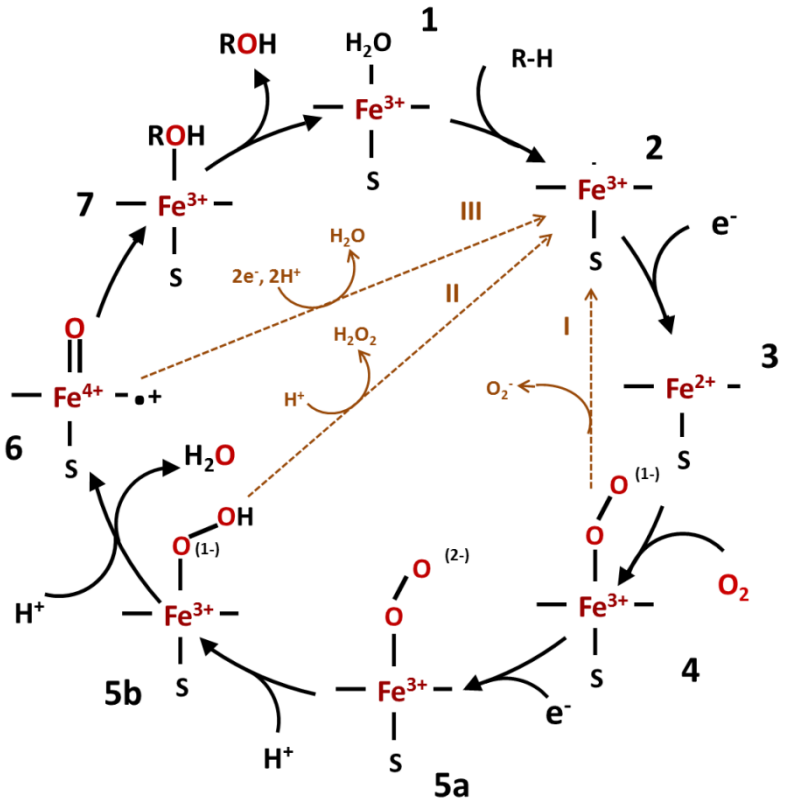

Figure 2: **Catalytic cycle of cytochromes P450**. Schematic overview of the catalytic P450 cycle adapted from Makris et al. [29] and Denisov et al. [28].

Being a heme-thiolate protein gives unique spectral properties and, reduced and in complex with CO, a typical absorption maximum at 450 nm. Respecting their sequence, cytochromes P450 can be divided into families and subfamilies: with a sequence similarity of ≥ 40%, they belong to the same family and with a similarity of ≥ 55% to the same subfamily (Figure 1) [30]. They mainly catalyze hydroxylation reactions by cleavage and activation of inert oxygen, whereby one oxygen atom is used for the substrate hydroxylation while the other one is reduced to water [31]. The catalytic cycle involves the substrate binding and a subsequent reduction of the central heme-iron by the transfer of an electron, which leads to the binding of molecular oxygen. A second electron consecutively reduces the bound oxygen, whose distal atom is abstracted as water molecule after proton transfer. Eventually, the hydroxylated substrate is released from the active site (Figure 2)

[29]. Since most of the P450s are not able to abstract electrons for the catalytic cycle from reduction equivalents, a successful reaction is dependent on electron transfer proteins, which are able to receive electrons from NADH or NADPH. The redox system either consists of one or of two proteins, and to date ten typical cytochrome P450 systems are reported, but the number is growing rapidly [32]. In the human body, class I and class II of the P450 systems are present.

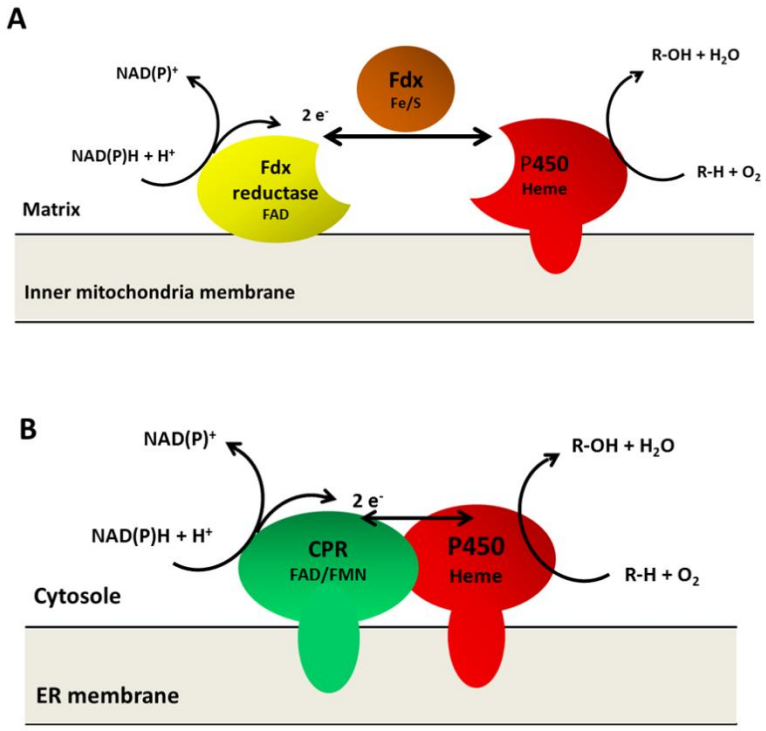

Figure 3: **Cytochrome P450 redox systems. A** The mitochondrial redox chain belongs to the class I redox systems and consists of the respective CYP, a ferredoxin reductase, designated as adrenodoxin reductase (AdR), and a ferredoxin, designated as adrenodoxin (Adx). AdR is associated with the inner mitochondrial membrane and receives electrons from NADPH, which subsequently are transferred to the CYP by the soluble Adx. **B** The two-component class II redox system contains the membrane-bound NADPH-dependent cytochrome P450 reductase (CPR) and the respective microsomal CYP. The electrons needed for the reaction are transferred to the FAD cofactor of the CPR and forwarded to the FMN domain, which interacts with the CYP after a conformational change. Figures according to [32].

Class I CYP systems contain two electron transfer proteins: a ferredoxin reductase with FAD as prosthetic group, which can either be membrane associated in case of mammals or soluble in case of bacteria, and a soluble ferredoxin with an iron-sulfur cluster as cofactor. The mitochondrial redox system involved in steroid hormone biosynthesis, which consists of the adrenodoxin reductase (AdR) and adrenodoxin (Adx), belongs to class II CYP systems (Figure 3 B) [33]. Class II CYP systems only appear in eukaryotes and possess one single electron transfer protein, a NADPH-dependent cytochrome P450 reductase. This protein contains two domains, one with FAD as cofactor, which receives electrons from NADPH, and the other one embeds FMN that interacts with the P450 after conformational changes (Figure 3A) [34, 35]. The fact that cytochromes P450 need redox transfer proteins hamper their application in industrially relevant biocatalytic processes, as a sufficient electron supply must be ensured for an efficient biotransformation [36, 37]. The next chapter will discuss limitations, but also promising perspectives for an application of P450s in biocatalysis.

1.3 P450s as versatile biocatalysts

Aside from the ability to perform substrate hydroxylations, cytochromes P450 also catalyze other reactions, like halogenation, epoxidation and even a chemically difficult to perform C-C bond cleavage [36, 37]. Their broad substrate range is as diverse as the existing P450 isoforms and reach from terpenoids and isoprenoids to fatty acids and steroids. Furthermore, P450s convince by their high stereo- and regioselectivity, which minimizes by-product formation. Aside from well-known ones, newly characterized orphan P450s reveal new exciting activities, which show the high potential of this protein family for an application in biocatalysis, which could replace established chemical synthesis procedures. In 2010, the CYPome of the myxobacterium *Sorangium cellulosum* So ce56 was elucidated, which consists of 21 open reading frames encoding for P450s [21]. In context of a screening of possible substrates, it was figured out that CYP267B1 is able to convert the anti-cancer drug epothilone B to 7-ketone epothilone B – a completely new epothilone B derivative, whose pharmaceutical potential further has to be elucidated [38]. In 2013, Paddon and coworkers could publish a biocatalytic process for the production of artemisinic acid, the precursor of the antimalarian drug artemisinin.

Artemisinin is synthesized in the plant *Artemisia annua* and its application as powerful drug is hindered by altering production yields as well as fluctuating costs, since it has to be obtained out of the plant material. With the help of synthetic biology and metabolic engineering, a *S. cerevisiae* strain was developed which overexpresses the enzymes involved in the pathway of natural artemisinin synthesis in *A. annua* with high yield of artemisinic acid [39, 40]. The rate-limiting step is catalyzed by the amorphadiene oxidase CYP71AV1, which belongs to the highly conserved protein family of cytochromes P450 [41]. Its overexpression strongly enhanced the catalytic efficiency and is crucial for the established process. But also with regard to today´s well established synthesis of pharmaceutically important glucocorticoid hydrocortisone, cytochrome P450 play a crucial role. Parts of the synthesis are performed in the fungal host *Curvularia lunata*, whose P450 systems are able to catalyze the final 11β-hydroxylation gaining hydrocortisone [42, 43]. In 2003, a *Saccharomyces cerevisiae* strain was published which is able to produce the hydrocortisone from a simple carbohydrate source by metabolic engineering [44]. For this, the yeast´s ergosterol biosynthesis pathway was modified to provide a steroidal substrate for CYP11A1, which catalyzes the first step of natural hydrocortisone synthesis by a side-chain cleavage of cholesterol. Furthermore, CYPs which are crucial for steroidogenesis in mammals, CYP21A2, CYP17A1 as well as CYP11B1, could successfully be overexpressed mimicking a mammalian pathway which was encoded by genomic integration as well as by vectors. Especially the biocatalytic ability of CYP21A2 will be an important issue later in this thesis. These two processes are impressing examples for modern synthetic biology and show the impact and the convenient application of P450s within biocatalytic systems to produce substances of high interest. Though these examples prove the fundamental suitability of cytochromes P450 for a biocatalytic application, some principle hurdles first have to be overcome. Limitations like a lack of enzyme stability and a low-yield heterologous protein expression can partially be minimized by the use of an appropriate expression host in combination with adapted expression conditions. Furthermore, the enzyme stability can be improved by biotransformation with whole cells, avoiding a degradation of the enzyme during a purification process. In recent years, modification of the primary enzyme structure led to an enhanced expression of mammalian CYPs in the host *Escherichia coli* by removal and replacement of the membrane-interacting domains, additionally leading to an improved stability [45]. One further

challenging bottle-neck regarding P450 mediated catalysis is definitely the sufficient supply with electrons for the reaction. Since P450s are external monooxygenases, they are dependent on electron transfer proteins, as shown in chapter 1.2. With regard to a biotechnological application, this means the expression and purification of one or two more proteins in case of *in vitro* applications or, concerning whole-cell catalysis, an enhanced metabolic burden for the host [46]. Additionally, the costly reducing equivalent NAD(P)H has to be abundant for a flowing electron supply, whereby whole-cell biocatalysis has to be preferred towards *in vitro* applications, since the host itself is able to serve as donor. An inefficient electron transfer to the CYP not only decelerates the biocatalysis, but also leads to an uncoupling of electrons, which subsequently leads to the formation of reactive oxygen species (ROS), as shown in Figure 2 [36, 47, 48]. An accumulation of ROS leads to an irreversible damage of the prosthetic heme group and, thus, to a decrease of active CYP, which consequently impedes the biocatalytic ability. Aside from the above-mentioned limitations, P450s in general exhibit very low turnover numbers and a low biocatalytic activity, which is not convenient for an industrially relevant biocatalytic process. Taken together, these bottlenecks are challenging with regard to a future biotechnological application of CYPs, but in the course of modern techniques in recent decades, a significant improvement of P450 systems is likely to be approximated [49]. Modern molecular evolution is based on three common techniques: random mutagenesis by using error-prone PCR addressing the whole protein sequence, site-directed mutagenesis, which goes along with saturation mutagenesis, and gene shuffling, which mimics sexual evolution in nature. Reetz et al. reviewed the achievements of molecular P450 evolution towards an application in organic synthesis by the example of P450-BM3 (CYP102A1) from *Bacillus megaterium*, which exhibits a significantly higher biocatalytic activity compared with other CYPs. The higher turnover number is due to the fact that CYP102A1 is a fusion protein consisting of a P450 domain and a CPR domain, which enables a direct electron transfer and minimizes an uncoupling [50]. Therefore, this enzyme is of great interest for an industrial application and was object of mutagenesis in last years to change regio-, stereo-, and enantioselectivity or to create new non-natural reactions. Recently, a CYP102A1 mutant was published, which is able to carry out an aziridination reaction having no counterpart found in nature yet – an impressive example for the potential of enzyme engineering and manipulation through molecular evolution

[51]. Aside from new reactions and changes in selectivity, it is of great interest to improve the catalytic activity of established CYP-mediated reactions, since most of them have poor turnover numbers. For this, one of the most important aspects in context of molecular evolution is a suitable and reliable screening system, which ensures a high-throughput screening of mutant libraries focusing on the improvement of the respective enzyme property with an appropriate enzyme expression as well as a sufficient host cell density. In view of a highly stereo- and regioselective substrate conversion, mammalian cytochromes P450 are of increasing interest in recent years. Particularly with respect to the hydroxylation of steroids, steroidogenic CYPs are suitable for the production of anti-inflammatory glucocorticoids, since a chemically performed hydroxylation exhibits a poor overall yield, deals with toxic compounds and forms many unwished by-products. In case of the *S. saccharomyces* strain, which was developed for a biocatalytic hydrocortisone production, many CYPs participating at the steroid biosynthesis are inserted into the yeast to mimic the natural synthesis pathway. The impact of natural and synthetic glucocorticoid on human and animal health as well as the role of the steroidogenic 21-hydroxylase (CYP21A2) in this context are treated in the next section.

1.4 Steroidogenic cytochromes P450 and the production of pharmaceutical glucocorticoids

Natural and synthetic glucocorticoids are of high pharmaceutical interest due to their anti-inflammatory and immunosuppressive effects. The most important natural glucocorticoid is cortisol (hydrocortisone), which is synthesized originating from cholesterol through a multi-step CYP-mediated reaction cascade in the *Zona fasciculata/reticularis* of the adrenal glands. The final step of cortisol synthesis is catalyzed by the 11β-hydroxylase CYP11B1, whose expression is controlled by ACTH (adrenocorticotropic hormone) and a cAMP regulated signaling pathway [52]. When cortisol binds to the respective glucocorticoid receptor (GR), it initiates an extensive stress response, which includes a change in glucose metabolism including the upregulation of the gluconeogenesis and an inhibition of fat metabolism [53-55]. On the other hand, cortisol secretion leads to a suppression of the immune response, especially of the inflammatory response, e.g. by inhibiting the transcription factor NF-κB, which is responsible for the transcriptional activation of cytokines [56]. The recognition that one is able to use cortisol as an anti-inflammatory drug happened over fifty years ago, when researches applied an adrenal cortical steroid extract to a patient with rheumatic arthritis and observed a beneficial effect on the symptoms. From there on, the industrial production of cortisol and its clinical application has been started. The cortisol binding to the GR is mainly mediated by its interaction of the hydroxyl groups at carbon atoms 11, 17 and 21, which are determined to be crucial for glucocorticoid action [57]. Since GR and the mineralocorticoid receptor (MR) possess highly similar structures and, therefore, cortisol also acts as mineralocorticoid due to its affinity to the MR, new synthetic glucocorticoids were synthesized originating from cortisol, which have lower mineralocorticoid effects and higher anti-inflammatory properties to reduce unwished side effects during treatment like a disturbance of the mineral household [58-60]. The most crucial change in the cortisol structure for a higher drug activity was the introduction of the 1,2-dehydrogenation in the steran A ring resulting in prednisolone. Furthermore, this simple dehydrogenation led to a decreased affinity to the MR and thus, to a decreased mineralocorticoid activity (Figure 4, Table 1) [61].

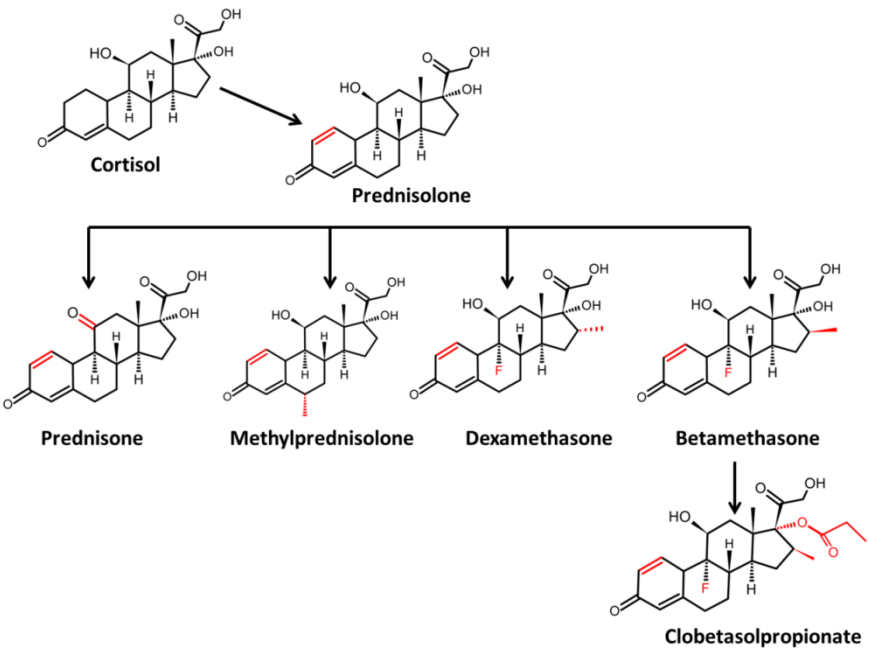

Figure 4: Overview of common pharmaceutical glucocorticoids. Synthetic glucocorticoids originate from the cortisol structure and all are modified by a 1,2-dehydrogenation in the steran A ring, which increases the drug efficiency. Additional halogenations and methylations further enhance the interaction with the glucocorticoid receptor and lower the mineralocorticoid action.

In the following years, prednisolone was further modified with additional methyl groups (C16: beta-/dexamethasone; C6: methylprednisolone) (Figure 4, Table 1). A total depletion of mineralocorticoid activity was achieved by halogenation of the steran skeleton, resulting in the highly active long-term steroids beta- and dexamethasone, to mention only the most important ones. Depending on the severity of the disease, synthetic steroids like cortisol are applied ectopically in the short-term treatment of dermal allergic reactions, while long-term glucocorticoids like beta- and dexamethasone serve as drugs applied systematically. The chemical production of glucocorticoids and mainly the introduction of hydroxylations into the steran skeleton at the crucial positions 11 and 21 consist of a complicated multi-step synthesis. It exhibits not only a poor selectivity, which leads to many by-products and, therefore, to several purification steps, but also involves toxic compounds like iodine [62-64]. Another very popular and demanded synthetic glucocorticoid represents methylprednisolone (medrol), which originates from prednisolone and possesses an

additional methylation at carbon atom six. It exhibits a 5 fold higher anti-inflammatory activity than cortisol and is a convenient drug in the treatment of autoimmune diseases like multiple sclerosis and of rheumatic arthritis (Figure 4, Table 1) [65, 66].

Table 1: Important glucocorticoids and their drug potency. Glucocorticoids originating from cortisol are listed in ascending order concerning their anti-inflammatory potency, the mineralocorticoid potency and their biological half-life. (According to [55])

	Anti-inflammatory potency	Mineralocorticoid potency	Biologic half-life [h]
Cortisol	1	1	8-12
Prednisolone	4	0.8	12-36
Prednisone	4	0.8	12-36
Methylprednisolone	5	0.5	12-36
Betamethasone	25	0	36-72
Dexamethasone	25	0	36-72

Methylprednisolone is produced by a chemical multistep-reaction with a crucial 21-hydroxylation at the end, resulting in premedrol, which is subsequently 1,2-dehydrogenated to medrol by a well-established microbial biocatalysis. With regard to a biocatalytic alternative for the selective introduction of an 11- and 21-hydroxylation, the most logical procedure is to apply enzymes, which are responsible for the synthesis of natural glucocorticoids, the steroidogenic cytochromes P450. The only enzyme so far known to perform a highly selective 21-hydroxylation, is the mammalian 21-hydroxylase, CYP21A2, which is involved in the cholesterol-originating steroid biosynthesis and provides the precursors for cortisol and aldosterone, the most important glucocorticoid and mineralocorticoid, respectively, by a 21-hydroxylation of the natural substrates progesterone and 17OH-progesterone (Figure 5) [67, 68].

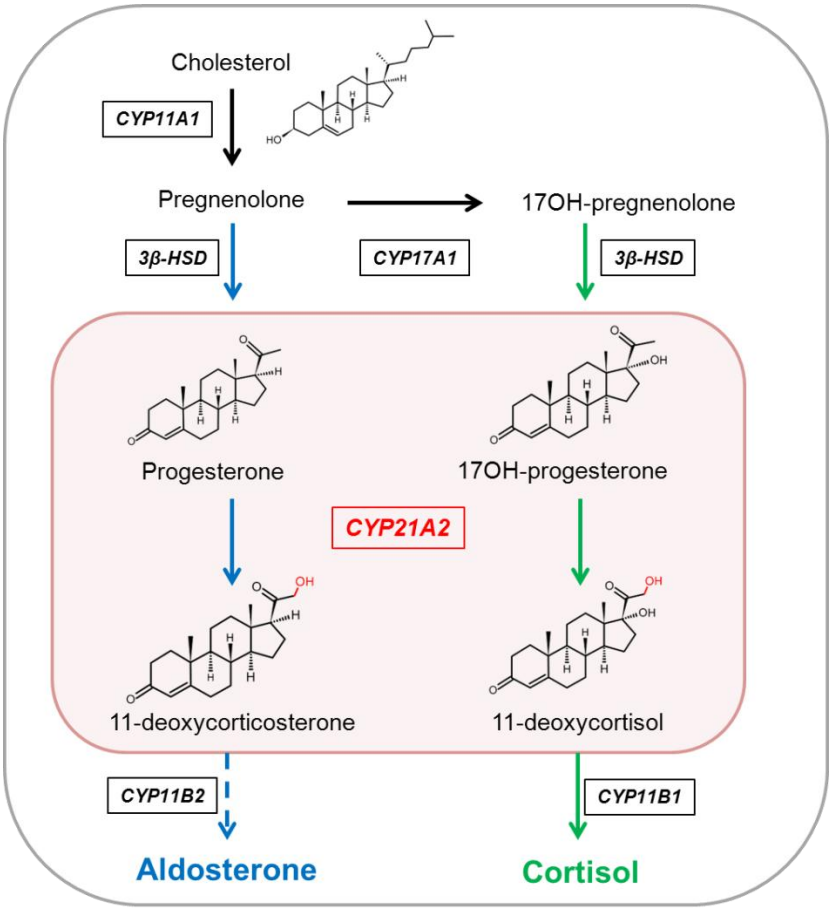

Figure 5: **CYP21A2-dependent biosynthesis of aldosterone and cortisol.** CYP21A2 participates in the biosynthesis of the main mineralocorticoid aldosterone and the main glucocorticoid cortisol from cholesterol by a selective hydroxylation of progesterone and 17OH-progesterone at carbon atom 21 to the respective precursor 11-deoxycorticosterone and 11-deoxycortisol.

CYP21A2 is expressed in the adrenal cortex, inserted into the endoplasmic reticulum membrane and represents, together with its natural redox partner CPR, a cytochrome P450 system class II. In human, the 21-hydroxylase is encoded on chromosome 6 and its transcription is regulated by ACTH. Near by the *CYP21A2* gene locus a second *CYP21* encoding sequence is located, which represents the pseudogene *CYP21A1*. The existence of this pseudogene is supposed to be responsible for many severe mutations within the *CYP21A2* gene due to illegitimate crossing over events [69]. These mutations are responsible for 95% of cases of congenital adrenal hyperplasia (CAH), which results in an impaired cortisol and aldosterone production and, therefore, in an accumulation of androgens. For a long time the impact of mutations on the structural as well as enzymatic

properties of CYP21A2 could not be elucidated, since an enzyme production in sufficient amounts by heterologous expression and in a suitable host was not possible. Although protein synthesis was possible in yeast species, the expression yield was to poor for downstream processing. When expression in the bacterial host *Escherichia coli* was enabled by a cleavage of the hydrophobic membrane anchor of bovine CYP21A2 to improve the solubility, the expression yield was high enough to examine CYP21A2 on a molecular level regarding its kinetics and to elucidate the crystal structure [70]. Furthermore, efforts were done to use the 21-hydroxylase as a biocatalyst in the host *Schizosaccharomyces pombe*, but due to a low expression yield the biocatalytic efficiency of this system was too poor for a valuable conversion of the natural substrates progesterone and 17OH-progesterone [71, 72]. For this reason, the powerful ability of CYP21A2 to perform a highly selective 21-hydroxylation was out of focus. The 11β-hydroxylase (CYP11B1), which catalyzes the last step in cortisol synthesis, is a mitochondrial cytochrome P450 belonging to the redox system class I. Similar to CYP21A2, its biocatalytic application was restricted to a poor expression yield due to its membrane protein character. The successful expression in *E. coli* was the first step initiating a biocatalytic application for cortisol production [73]. This state of art represents the initial situation of this work, which focusses the biotechnological application of steroidogenic CYPs.

1.5 Aim and scope of this work

The biotechnological application of steroidogenic cytochromes P450 is of great interest for the introduction of crucial hydroxyl groups into pharmaceutically relevant glucocorticoids, since in the long term the current chemical hydroxylation should be replaced by a sustainable and more selective biocatalytic process. CYP-dependent biocatalysis is restricted by poor turnover numbers, which can be overcome by a molecular evolution combined with a suitable screening system. In this work, the recent progress in using *E. coli* as expression host for mammalian CYPs was taken up to establish whole-cell systems for the CYP21A2 mediated 21-hydroxylation of medrane to premedrol, the precursor of methylprednisolone.

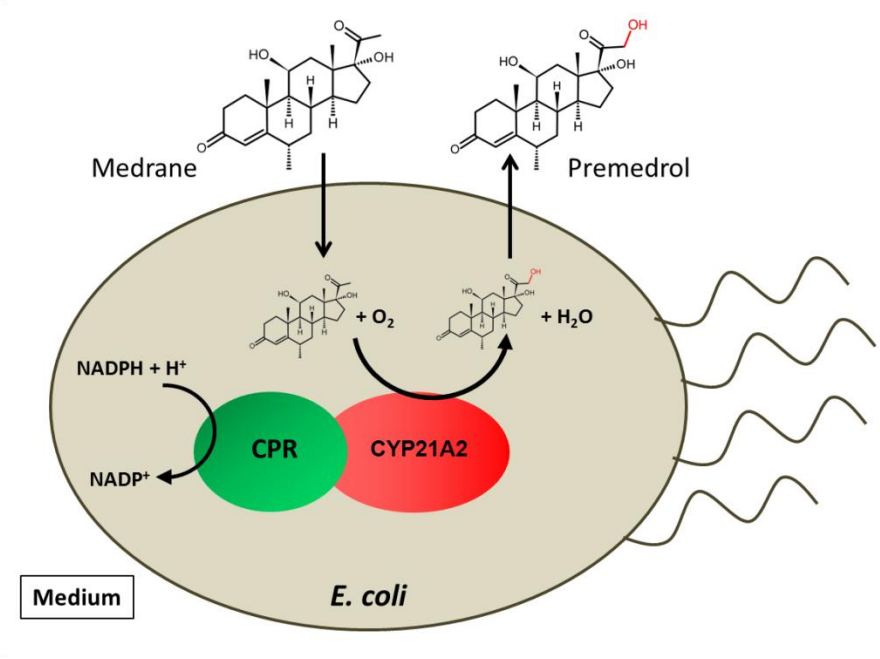

Figure 6: **Scheme of a CYP21A2 based whole-cell system in *E. coli*.** Schematic representation of a medrane-converting *E. coli* whole cell system based on a heterologous expression of bovine CYP21A2 and CPR as redox transfer protein.

Additionally, CYP11B1- and CYP11B2-based whole-cell systems were established for the production of important metabolites as well as pharmaceutically relevant steroids. Furthermore, a convenient screening host for molecular evolution should be generated to facilitate cultivation and biotransformation in a microtiter scale.

2. Scientific articles

The obtained results presented in this work are published in the articles listed below:

2.1 Simone Brixius-Anderko et al. (2015)

A CYP21A2 based whole-cell system in *Escherichia coli* for the biotechnological production of Premedrol

Simone Brixius-Anderko**,** Lina Schiffer, Frank Hannemann, Bernd Janocha, Rita Bernhardt

Microbial Cell Factories, 2015, 14:135 doi: 10.1186/s12934-015-0333-2

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Patent with Sanofi Aventis, "21-Hydroxylation of Steroids";
Submission number 1000263078
Application number EP14306740.3
Submission: 30 October 2014

Brixius-Anderko *et al. Microb Cell Fact (2015) 14:135*
DOI 10.1186/s12934-015-0333-2

MICROBIAL CELL FACTORIES

RESEARCH Open Access

A CYP21A2 based whole-cell system in *Escherichia coli* for the biotechnological production of premedrol

Simone Brixius-Anderko[1], Lina Schiffer[1], Frank Hannemann[1], Bernd Janocha[2] and Rita Bernhardt[1*]

Abstract

Background: Synthetic glucocorticoids like methylprednisolone (medrol) are of high pharmaceutical interest and represent powerful drugs due to their anti-inflammatory and immunosuppressive effects. Since the chemical hydroxylation of carbon atom 21, a crucial step in the synthesis of the medrol precursor premedrol, exhibits a low overall yield because of a poor stereo- and regioselectivity, there is high interest in a more sustainable and efficient biocatalytic process. One promising candidate is the mammalian cytochrome P450 CYP21A2 which is involved in steroid hormone biosynthesis and performs a selective oxyfunctionalization of C21 to provide the precursors of aldosterone, the main mineralocorticoid, and cortisol, the most important glucocorticoid. In this work, we demonstrate the high potential of CYP21A2 for a biotechnological production of premedrol, an important precursor of medrol.

Results: We successfully developed a CYP21A2-based whole-cell system in *Escherichia coli* by coexpressing the cDNAs of bovine *CYP21A2* and its redox partner, the NADPH-dependent cytochrome P450 reductase (*CPR*), via a bicistronic vector. The synthetic substrate medrane was selectively 21-hydroxylated to premedrol with a max. yield of 90 mg L^{-1} d^{-1}. To further improve the biocatalytic activity of the system by a more effective electron supply, we exchanged the CPR with constructs containing five alternative redox systems. A comparison of the constructs revealed that the redox system with the highest endpoint yield converted 70 % of the substrate within the first 2 h showing a doubled initial reaction rate compared with the other constructs. Using the best system we could increase the overall yield of premedrol to a maximum of 320 mg L^{-1} d^{-1} in shaking flasks. Optimization of the biotransformation in a bioreactor could further improve the premedrol gain to a maximum of 0.65 g L^{-1} d^{-1}.

Conclusions: We successfully established a CYP21-based whole-cell system for the biotechnological production of premedrol, a pharmaceutically relevant glucocorticoid, in *E. coli* and could improve the system by optimizing the redox system concerning reaction velocity and endpoint yield. This is the first step for a sustainable replacement of a complicated chemical low-yield hydroxylation by a biocatalytic cytochrome P450-based whole-cell system.

Keywords: Methylprednisolone, Medrane, CYP21A2, Cytochrome P450, Whole-cell biocatalysis, *E. coli*, Etp1^{fd}, Arh1, CPR, Adx, Steroid

Background

Since the 1950's, the development of synthetic glucocorticoids is of growing interest with the aim to substitute the natural steroid hormone hydrocortisone as therapeutical compound. The superficial aim is to reduce severe hydrocortisone induced side effects, such as the disturbance of the electrolyte homeostasis, and to synthesize molecules with increased anti-inflammatory effects [1]. Based upon the artificial hydrocortisone derivative prednisolone the highly effective compound medrol (6-methylprednisolone) was developed by addition of a methyl group at carbon atom 6. Medrol turned out to have a far higher glucocorticoid activity than hydrocortisone without a comparative increase of electrolyte

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activity [2]. Medrol was synthesized from its precursor premedrol via a simple to perform biotechnological 1,2 dehydrogenation [3]. Today, medrol is a widespread drug in the treatment of autoimmune diseases, allergic reactions, multiple sclerosis and rheumatic arthritis [4]. Therefore, the demand for this pharmaceutically highly relevant glucocorticoid is still increasing. One bottleneck during contemporary premedrol and, therefore, medrol production is the hydroxylation of carbon atom 21. The chemical introduction of a hydroxyl group into a steran scaffold consists of a multistep-synthesis with the necessity to apply protecting groups and toxic compounds like iodine [5]. As by-products occur after each reaction step, a time consuming chromatographic purification is necessary, which leads to a reduced overall yield and a low efficiency factor [6, 7]. With regard to a more sustainable and less polluting production process and a regio- and stereoselective oxyfunctionalization at C21, the focus has shifted from the chemical process to an enzyme based biotechnological production of medrol and its precursor. A promising candidate for the enzymatic reaction is the mammalian cytochrome P450 21-hydroxylase (CYP21A2), which is a member of the cytochrome P450 (CYP, P450) superfamily. CYP21A2 is a protein of the endoplasmic reticulum and plays a crucial role in steroid hormone biosynthesis by providing the precursors of the most important mineralocorticoid, aldosterone, and the main glucocorticoid, cortisol, via a highly selective 21-hydroxylation, which is ensured by a unique amino acid arrangement within the active site [8–10]. A sufficient electron supply for the hydroxylation reaction is realized by its natural redox partner, the NADPH-dependent cytochrome P450 reductase (CPR), a membrane bound protein as well [11, 12]. Cytochromes P450 are external monooxygenases and exhibit, when reduced and in complex with CO, a unique absorption maximum at 450 nm due to the cysteinate coordinated heme group at the active site [13]. Their ability to functionalize molecular oxygen empowers them to catalyze a broad range of reactions, such as hydroxylations and even a chemically difficult to perform C–C bond cleavage. Apart from steroid hormone biosynthesis, they act as main detoxifying enzymes in the liver and are, therefore, involved in xenobiotics and drug metabolism. P450s are able to convert a great variety of substrates like steroids, terpenes as well as fatty acids, which shows their high potential as versatile biocatalysts [14, 15]. Since the 1960s, cytochromes P450 are crucially involved in the glucocorticoid synthesis in large scale by fermentation of species of the fungus *Curvularia,* whose later characterized P450 system was shown to be able to convert 11-deoxycortisol to cortisol [16–18]. In 2003, the application of a modified *Saccharomyces cerevisiae* strain was published, which performs cortisol production from a simple carbon source [19]. Aside from genetic manipulation of the yeast's ergosterol synthesis pathway, CYPs involved in steroid hormone synthesis, including CYP21A2, were expressed in this yeast strain, which shows the high importance of these enzymes for stereo- and regioselective steroid hydroxylation. To date, CYPs find their application in various industrial production processes and the number is still growing. Efforts are made to design whole-cell systems with single CYPs for the desired reaction in a suitable host to avoid by-products originating from homologous CYP systems like in case of *C. lunata*. Previously, a heterologous human CYP11B1 whole-cell system for a more selective cortisol production has been published [20]. Biocatalysis with whole cells ensures a better protein stability and a supply with costly cofactors, such as NADPH [21]. Concerning mammalian CYP21A2, a whole-cell system in the yeast *Schizosaccharomyces pombe* was already established with human CYP21A2, but with limited success due to a low recombinant protein yield and the host's long lasting generation time [22]. In other approaches to develop CYP based whole-cell systems, *Escherichia coli* emerged to be a suitable host attributed to its short generation time and the lack of intrinsic CYP systems [23]. Functional bovine CYP21A2 could already be successfully expressed with high yield in *E. coli* [24, 25]. These fundamentals were the starting point for our efforts to establish an efficient CYP21A2-based whole-cell system in *E. coli* for the production of premedrol, the precursor of medrol, via a simple one-step hydroxylation at C21. In the following, we demonstrate the successful expression and purification of bovine CYP21A2 and in vitro studies concerning the substrate-protein-interaction, the development of a biotransformation in whole cells and an improvement of the biocatalytic efficiency by using alternative redox systems for a more sufficient electron supply.

Results and discussion

Protein purification and in vitro characterization

Purification of bovine CYP21A2

Since bovine CYP21A2 could already be expressed in *E. coli*, we chose this mammalian CYP21A2 isoform for the initial examination of its suitability for a whole-cell system in *E. coli.* To ascertain whether bovine CYP21A2 is able to convert medrane to premedrol by a stereoselective 21-hydroxylation, the protein had to be expressed and purified according to Arase et al. [24]. The cDNA was subcloned into a pET17b vector, resulting in the vector pET17b_21b. The vector was co-transformed with the vector pGro12, which encodes for the *E. coli* chaperones GroEL/ES, into C43(DE3) cells. After protein

expression, cell lysis took place via sonification for the subsequent purification. The purification was performed via IMAC, anion and cation exchange chromatography and the protein was analyzed by SDS-PAGE, confirming the estimated molecular weight of 54.6 kDa, and by CO difference spectroscopy, which confirmed a correct insertion of the heme prosthetic group by showing a typical absorption maximum at 450 nm without a hint of inactive protein, indicated by a peak at 420 nm (Fig. 1). Taken together, the expression as well as the purification of bovine CYP21A2 was successful with an expression level of max. 398 nmol L^{-1} culture. The purified enzyme was used for further investigations.

In vitro conversion of medrane with purified CYP21A2

In order to prove a selective conversion of medrane to premedrol by a 21-hydroxylation, in vitro assays were carried out to perform a proof-of-principle, as medrane exhibits slight modifications within the steran skeleton compared with the natural CYP21A2 substrates progesterone (P4) and 17OH-progesterone (17OH-P4). Hence, substrate conversion with purified bovine CYP21A2 was performed with the synthetic substrate medrane characterized by its additional methyl group at carbon atom 6 and a hydroxyl group at carbon atom 11 compared with the natural substrate 17OH-P4. HPLC analysis revealed a 21-hydroxylation of medrane and demonstrated a stereoselective production of the wished product premedrol in an efficient biocatalytic one-step hydroxylation without by-product formation (Fig. 2b). It has been shown that bovine CYP21A2 is able to hydroxylate a synthetic substrate of high pharmaceutical interest.

Development of a whole-cell system for a biocatalytic premedrol production

After a conversion of medrane to premedrol by purified CYP21A2 was verified in vitro, the subsequent experiments focused on an establishment of a biotransformation using whole cells, showing advantages like an improved enzyme stability and the supply of costly co-factors by the cell itself [21]. For the development of a whole-cell system, the bicistronic vector p21b_bRED was constructed, carrying the cDNAs for bovine *CYP21A2* and *CPR* (Fig. 3). The natural redox partner CPR is responsible for electron supply in the endoplasmic reticulum through protein interaction. Cells were co-transformed with the respective vector and with the plasmid pGro12, encoding the chaperones GroEL/ES to ensure a proper folding of the membrane proteins CYP21A2 and CPR [26]. Although complex medium is suitable for bacterial cell growth and supports a high expression yield of recombinant proteins, it is inappropriate for whole-cell biotransformations with cytochromes P450 due to inhibitory effects of medium compounds and *E. coli* metabolites such as indole [27, 28]. For this reason, biotransformation with whole cells was performed with resting cells using potassium phosphate buffer as a conversion medium. As the metabolism, including protein biosynthesis, of resting cells is reduced to a minimum, more co-factors like NADPH can be recruited for the CYP dependent reaction [29]. By the addition of glycerol to the reaction mix, an NADPH-regeneration is ensured by the activity of metabolic enzymes like the isocitrate dehydrogenase. For initial examination, medrane was added to the whole-cell system and samples were taken after 24 h for HPLC analysis. Medrane was converted to premedrol by the constructed system without

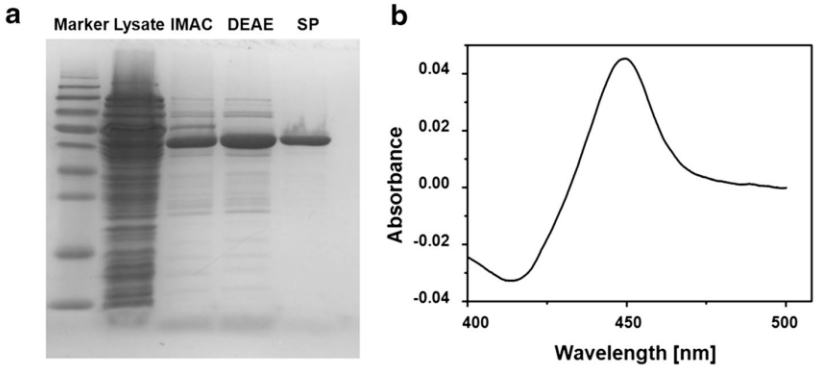

Fig. 1 Protein analysis of purified bovine CYP21A2 after three chromatographic steps. **a** SDS-PAGE analysis and protein staining shows the purification steps of CYP21A2 via IMAC, DEAE- and SP-Sepharose resulting in a single protein band with a size of approx. 55 kDa (estimated weight: 54.6 kDa); **b** Difference spectroscopy of purified bovine CYP21A2 was performed showing a typical absorption maximum at 450 nm in the reduced state in complex with CO

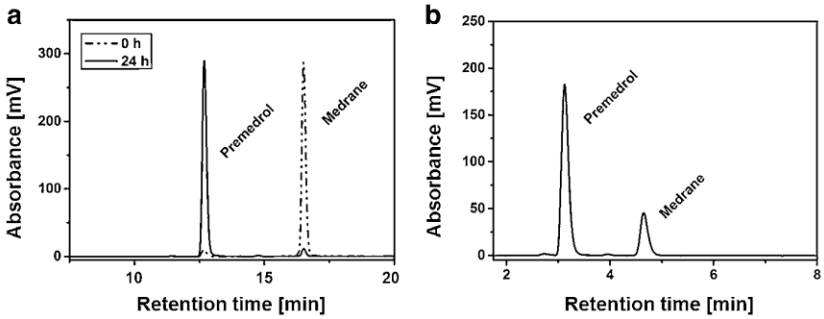

Fig. 2 HPLC chromatograms of the in vivo and in vitro conversion of medrane with bovine CYP21A2 and CPR. **a** Medrane was converted with resting cells of C43(DE3) containing the bovine CYP21A2 isoform and CPR encoding vector p21b_bRED. Samples were taken after 24 h and extracted for HPLC analysis. The steroids were separated by an acetonitril:water gradient. **b** Substrate conversions of medrane were performed with purified bovine CYP21A2 and its redox partner CPR for 30 min. Steroids were extracted and analyzed by HPLC to verify a selective conversion of medrane to premedrol. The steroids were separated by an acetonitril:water gradient, showing the 21-hydroxylated product premedrol

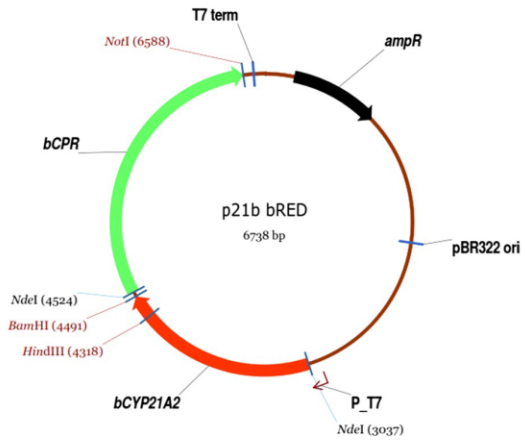

Fig. 3 Vector map of p21b_bRED containing the bicistronic transcription unit for bovine CYP21A2 and the CPR. For the whole-cell biotransformation, this bicistronic vector was constructed, based on the pET17b vector with a T7 promoter and an ampicillin resistance gene. The *CPR* cDNA encodes for the full length protein

by-product formation, verifying the highly specific 21-hydroxylation not only with purified enzymes but also by a biotransformation with whole cells (Fig. 2a). With a substrate concentration of 250 µM, a maximum premedrol yield of 93 mg L^{-1} d^{-1} could be achieved. Since the human CYP21A2 isoform shares a 79 % sequence homology to the bovine one, we additionally tested the human CYP21A2 isoform concerning its ability to produce premedrol. Just as the bovine enzyme, it performs a selective 21-hydroxylation of medrane, but exhibits a poor yield with 40 mg L^{-1} d^{-1} (Additional file 1: Fig. S1). Therefore, we concentrated on the bovine isoform in further experiments to improve the whole-cell system's efficiency.

Improvement of the whole-cell system by using different redox partners

CYP involved whole-cell systems require an efficient cytochrome P450/redox partner interaction to ensure a sufficient electron supply for the hydroxylation reaction [15]. Therefore, a stabilized redox partner synthesis as well as an optimal interaction with the CYP should be intended. In the following, we focused on these aspects by testing various redox systems of different origin.

Selection of alternative redox partners for CYP21

Bovine membrane-bound microsomal CPR represents the naturally occurring redox partner for CYP21 in the endoplasmic reticulum and was therefore the first choice for a co-expression in a whole-cell system. Though, this protein is difficult to produce recombinantly due to its property as a membrane protein. Therefore, efforts were undertaken to search for alternative redox partners of the bovine CYP21A2, which are easily expressed in *E. coli* in sufficient amounts, more stable and solvent resistant. On this account, we concentrated on the participating proteins of three naturally occurring redox systems, each consisting of two components, a ferredoxin or flavodoxin reductase and a ferredoxin as final electron donor for CYP21A2.

First, we focused on the soluble ferredoxin adrenodoxin (Adx), which reconstitutes the mitochondrial electron transfer system together with the membrane associated adrenodoxin reductase (AdR), which receives electrons from NADPH [12, 30–32]. This system is responsible for the electron supply of the mitochondrial

CYPs, CYP11A1, CYP11B1 and CYP11B2, which are also involved in steroid hormone biosynthesis. Pechurskaya et al. showed, that Adx is able to transfer electrons to purified CYP21A2, in the case of truncated CYP21A2 even more effectively than the CPR in in vitro assays [33]. In this work, we used the Adx version, Adx_{1-108}, which exhibits an increased electron transfer efficiency to some CYPs [34, 35].

Second, a redox system originating from the fission yeast *S. pombe* and consisting of the adrenodoxin reductase homologue 1 (arh1) and the ferredoxin domain of electron transfer protein 1 ($etp1^{fd}$) was considered, since Ewen et al. showed that this system is able to substitute Adx and AdR regarding an electron transfer to CYP11A1 [36]. In *S. pombe*, arh1 and $etp1^{fd}$ are involved in heme biosynthesis in the mitochondrium [37]. $Etp1^{fd}$ as iron-sulfur protein is highly homologous to adrenodoxin and is able to transfer electrons to mammalian steroidogenic CYPs [38, 39]. Here, we used the truncated version of $etp1^{fd}$ (516–618). Both proteins can be produced as cytosolic proteins in *E. coli*. Furthermore, it has been demonstrated, that arh1 can be reduced not only by NADPH like AdR, but also by NADH and that arh1 of *S. cerevisiae* is able to interact with bovine Adx [40]. Regarding a whole-cell system, a second electron pool could be of great advantage for a more efficient hydroxylation rate [36]. Janocha et al. already demonstrated a biotechnological application of arh1 and $etp1^{fd}$ from *S. pombe* with CYP105A1 from *Streptomyces griseolus* [41]. As in previous works of our laboratory, we used an arh1 variant with an improved FAD-binding behavior, ensuring co-factor stability [36].

Third, we applied the *E. coli* NADPH-flavodoxin reductase Fpr as an alternative reductase for a whole-cell system. It has been demonstrated previously that the soluble Fpr is able to transfer electrons to Adx and, therefore, represents an efficient substitution for AdR [27]. The Fpr together with flavodoxin A (FldA) is part of an *E. coli* redox system, which is involved in biosynthetic processes such as amino acid synthesis [42, 43].

To verify whether Adx and $etp1^{fd}$ as final electron transfer proteins are able to supply CYP21A2 with electrons, in vitro assays were carried out with different combinations of reductases and ferredoxins, listed in Table 1. HPLC analysis revealed, that both, Adx and $etp1^{fd}$, are able to transfer electrons to CYP21A2, no matter which reductase was chosen (Fig. 4). It was shown for the first time, that $etp1^{fd}$ is able to interact with CYP21A2 and, furthermore, to recruit the *E. coli* reductase Fpr as redox partner.

In the following, we show efforts to exchange the natural redox partner CPR by bovine Adx as well as $etp1^{fd}$ as final electron donors in combination with different reductases in a whole-cell system to achieve an enhanced premedrol yield.

Table 1 Vectors for the *E. coli* whole-cell system

Vector	Reductase	Origin	Ferredoxin	Origin
p21b_bRED	CPR	*Bos taurus*		
p21b_AdAx	AdR	*Bos taurus*	Adx	*Bos taurus*
p21b_ArAx	arh1	*S. pombe*	Adx	*Bos taurus*
p21b_FrAx	Fpr	*E. coli*	Adx	*Bos taurus*
p21b_ArEt	arh1	*S. pombe*	$etp1^{fd}$	*S. pombe*
p21b_FrEt	Fpr	*E. coli*	$etp1^{fd}$	*S. pombe*

Six pET17b based vectors were constructed, each carrying the *CYP21A2* cDNA, in either a bicistronic construct with the cDNA for *CPR* or in a tricistronic construct with the cDNAs for Adx_{1-108} or $etp1^{fd}$ as final electron donors. The origin of the respective protein is mentioned

Construction of vectors for a whole-cell system with various redox chains

We constructed three vectors with a tricistronic transcription unit, each containing Adx as final electron donor. The vector p21b_ArAx was constructed containing the ORF for bovine CYP21A2, followed by the ORF for bovine AdR and Adx_{1-108}, which represents the mitochondrial redox chain. Then, we replaced the AdR sequence with the one for arh1 from *S. pombe*. Finally, we used the *E. coli* reductase Fpr instead of AdR. The three resulting constructs are shown in Fig. S2 (Additional file 2).

Two more vectors were constructed, containing $etp1^{fd}$ as electron donor of CYP21A2, on the one hand in combination with its natural ferredoxin reductase from *S. pombe*, arh1 (p21b_ArEt), on the other hand with the *E. coli* reductase, Fpr (p21b_FrEt). The resulting constructs are shown in Fig. S3 (Additional file 3). All constructed vectors are listed in Table 1.

Evaluation of the CYP21A2 whole-cell systems with different redox partners

C43(DE3) cells were co-transformed with pGro12 and the constructed vectors, cultivated simultaneously to compare the initial productivity of each system as well as the endpoint yield of premedrol. Whole-cell biotransformation was carried out with resting cells in potassium phosphate buffer and samples for HPLC analysis were taken after 0, 2, 4, 6, 10 and 24 h to get a characteristic time course of the product formation depending on the respective redox system. A substrate concentration of 500 µM medrane was applied. HPLC analysis verified the biotransformation ability of each system. Regarding the endpoint yield, the systems containing the mitochondrial (AdR/Adx) and microsomal (CPR) redox partners produced the lowest amount of premedrol with 41 and

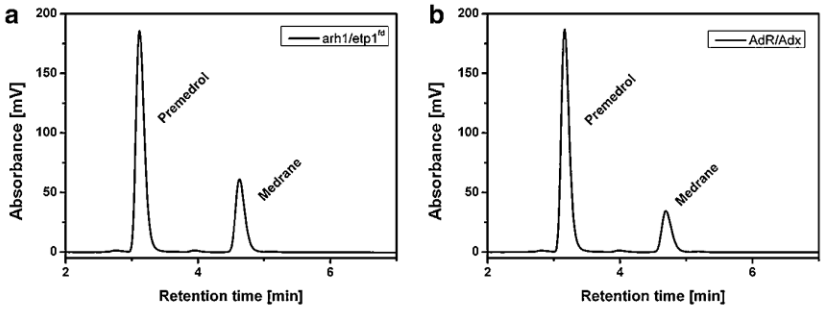

Fig. 4 HPLC chromatograms of the in vitro biotransformation of medrane with bovine CYP21A2 and Adx or etp1^{fd} as final redox partner. Substrate conversions of medrane were performed with purified bovine CYP21A2 and **a** Adx as final electron donor, here with its natural reductase AdR, and **b** etp1^{fd} as final electron donor, here with its natural reductase arh1 for 30 min. Steroids were extracted and analyzed by HPLC to verify a selective conversion to premedrol, respectively

87 mg L^{-1} d^{-1}, respectively. Taken the fact into account that AdR and CPR are membrane-associated and membrane-bound proteins, respectively, the recombinant synthesis and stability of these enzymes might be limited and, therefore, could represent a disadvantage for their application in a biotransformation process. Remarkably, the endpoint yield was dependent on the expressed reductases and not on the respective ferredoxin, suggesting that within this whole-cell system the functionality of the reductases is a limiting factor. The biotransformation overall yield with Fpr was higher than that with CPR with 127 and 115 mg L^{-1} d^{-1}, no matter which ferredoxin, Adx or etp1^{fd}, was the final electron donor for CYP21. The same was observed with arh1 as reductase with 156 mg L^{-1} d^{-1} together with Adx and 167 mg L^{-1} d^{-1} with its natural redox partner etp1^{fd}, emphasizing that the soluble proteins, Fpr and arh1, are more suitable for a whole-cell system (Fig. 5; Table 2).

Though the endpoint yields of the different systems are similar when using the same reductase, the time course revealed a crucial difference regarding reaction velocity. While the velocities of the redox partner combinations arh1/Adx, Fpr/Adx and Fpr/etp1^{fd} are similar within the first 4 h, the system containing the reductase as well as the ferredoxin from *S. pombe* exhibits a higher efficiency with about doubled product formation between 2 and 4 h of substrate conversion, obviously due to the fact that arh1 together with etp1^{fd} represents a natural redox chain with optimal protein–protein interaction properties. Table 2 lists the initial and final product formation rates.

Taken together, we clearly demonstrated, that a redox protein exchange for the CYP21 whole-cell system increased the overall yield from about 90 mg L^{-1} d^{-1} to about 167 mg L^{-1} d^{-1}, by use of arh1 and etp1^{fd} instead of CPR. It was also demonstrated that the reaction velocity

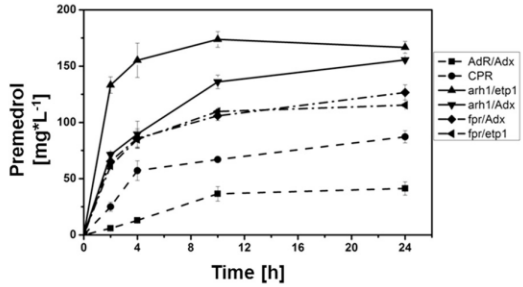

Fig. 5 Time-dependent premedrol formation by different CYP21A2 based whole-cell systems. Whole-cell biotransformation was performed with all constructed systems simultaneously by using resting cells. Samples were taken after 0, 2, 4, 10 and 24 h and analyzed via HPLC. The premedrol yield at several time points was determined for each system. All values represent the mean of triplicates with the respective standard deviation

is strongly dependent on the expressed redox proteins. With a substrate concentration of 1 mM medrane we could maximize the premedrol yield to 320 mg L^{-1} d^{-1} with the arh1 and etp1^{fd} redox chain in subsequent experiments. The fact that arh1 is able to receive electrons not only from NADPH but also from NADH (Additional file 3: Fig. S3) emphasizes the great potential of this reductase in a whole-cell application tapping an additional electron pool compared with the NADPH dependent Fpr and AdR, taking into account the fact that in *E. coli* NADH is the predominant co-factor under normal metabolic conditions of *E. coli* [44, 45].

To compare our established *E. coli* system with the human CYP21A2 based whole-cell system in *S. pombe*, we performed substrate conversions with the natural substrate 17OH-progesterone, since Zehentgruber et al. used it in an *S. pombe* whole-cell system [22]. With the *E.*

Table 2 Premedrol yield of different systems for whole-cell biotransformations performed with bCYP21A2 and various redox chains

Redox system	Overall yield premedrol (mg L^{-1} d^{-1})	Initial rate (mg L^{-1} h^{-1})
CPR	87 ± 5	13 ± 2
AdR/Adx	41 ± 6	3 ± 0.2
arh1/Adx	156 ± 2	36 ± 1
Fpr/Adx	127 ± 7	33 ± 2
arh1/etp1^{fd}	167 ± 5	67 ± 4
Fpr/etp1^{fd}	115 ± 1	31 ± 0.4

The premedrol overall yield was determined via HPLC analysis after 24 h conversion with all constructs simultaneously. The initial rate was calculated taking the first 2 h into account after starting the reaction by substrate addition. All values represent the mean of triplicates with the respective standard deviation

coli system we achieved 308 ± 16 mg L^{-1} d^{-1} of the product 11-deoxycortisol, which is an about fourfold higher product yield compared with the system in *S. pombe* producing 77 mg product per L and day. Taken into account that Zehentgruber et al. used a cell density of 360 g L^{-1}, which is tenfold higher than the applied *E. coli* density of 24 g L^{-1}, we achieved a productivity of 37 µmol g^{-1} cell wet weight, while only 0.625 µmol g^{-1} cell wet weight were produced with the *S. pombe* system, which is 60 times less. This data clearly demonstrates the high efficiency and productivity of the established *E. coli* whole-cell system.

To examine the stoichiometry of CYP21A2:arh1:etp1^{fd}, which are encoded by a tricistronic transcription unit, Western blot analysis was carried out for each enzyme after an expression time of 28 h according to Janocha et al. [41]. The highest expression level was determined for etp1^{fd} with approx. 880 nmol L^{-1}. The reductase arh1 expression level is estimated to be approx. 498 nmol L^{-1} and the lowest one is for CYP21A2 with approx. 119 nmol L^{-1}. Thus, the proteins are expressed with a ratio of 1:4:7 (CYP21A2:arh1:etp1^{fd}) demonstrating an excess of reductase and ferredoxin, which supports a sufficient electron supply to CYP21A2 and underlines the system's high efficiency (Fig. 6).

Determination of dissociation constants by difference spectroscopy

To compare the binding ability of bovine CYP21A2 to the synthetic substrate medrane to natural ones, progesterone and 17OH-progesterone, and to examine a possible limitation for medrane conversion due to a decreased protein binding, we determined the dissociation constant of the CYP21A2-medrane complex by difference spectroscopy. Complex formation between a potential substrate and CYP21A2 is spectroscopically detectable as type I shift due to the replacement of the heme coordinated H_2O molecule. Titration of CYP21A2 with increasing amounts of medrane shows a typical type I shift (Fig. 7a) and, therefore, underlines a medrane conversion by CYP21A2. The difference of the absorbance maximum and minimum plotted against the substrate concentration of each titrating step results in a hyperbolic regression curve revealing a K_D value of 11.27 ± 0.28 µM for medrane. To compare the K_D value

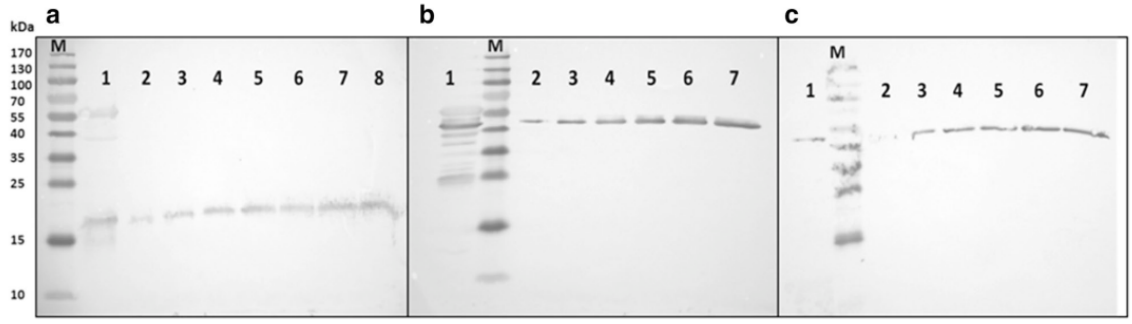

Fig. 6 Western blot analysis of CYP21A2 and redox proteins in the whole-cell system for determination of the stoichiometric ratio of the three proteins encoded by the tricistronic construct p21b_ArEt. Western blot analysis was performed with polyclonal antibodies against **a** etp1^{fd} (11.3 kDa), **b** arh1 (51 kDa), c bovine CYP21A2 (54.6 kDa). In each blot, lane 1 represents the *E. coli* cell extract expressing arh1, etp1^{fd} and CYP21A2 after 28 h. **a** *lane 2–8* represents purified etp1^{fd} in increasing amounts (10, 20, 30, 40, 50, 75 and 100 ng), **b**, **c** The *lanes 2–7* show purified arh1 and CYP21A2 in increasing amounts (arh1: 25, 50, 75, 125, 187.5 and 250 ng, CYP21A2: 24, 48, 95, 143, 191 and 239 ng). The with "M" marked *lanes* all represent a prestained protein marker. The relative lane intensities, which correlate with the respective protein masses, were determined and compared to the intensity of the whole-cell system sample. Mass values were converted into the amount of substances and extrapolated to the expression yield per liter culture. Note that etp1^{fd} is known to give a single band in the range of the double mass expected to see on the SDS-PAGE

of the non-natural substrate medrane with those of the natural substrates, 17OH-progesterone and progesterone, we additionally titrated CYP21A2 with increasing amounts of these steroids. Hyperbolic regression resulted in a K_D value for 17OH-progesterone of 0.14 ± 0.01 µM and for progesterone of 0.34 ± 0.01 µM (Fig. 7b, c), implicating a higher affinity of natural CYP21A2 substrates compared with the synthetic one, which is due to the amino acid arrangement in the active site of CYP21A2 to ensure a selective 21-hydroxylation of the natural substrates [10]. Considering the nearly 100-fold higher K_D value of medrane compared with the natural substrate 17OH-P4, an enzyme improvement could aspire a stronger binding of the synthetic substrate to promote a more efficient premedrol production. This hypothesis is underlined by a higher product formation when using 17OH-progesterone as a substrate displaying a yield of 889 ± 59 µM d^{-1} 11-deoxycortisol compared with a premedrol yield of 640 ± 13 µM d^{-1}. Hence, biotransformation with CYP21A2 using the natural substrate with the lowest dissociation constant shows a 40 % higher product formation than bioconversion with medrane.

Scale-up of the whole-cell system with CYP21A2 and the redox partners arh1 and etp1^{fd}

After the establishment of a CYP21A2 based whole-cell system and a further improvement of the system's efficiency by alternative redox chains in shaking flasks, we pursued a scale-up of the system by a fermentation approach with increased cell density and the possibility to supplement oxygen by the stir velocity, since a sufficient oxygen supply is indispensable for CYP

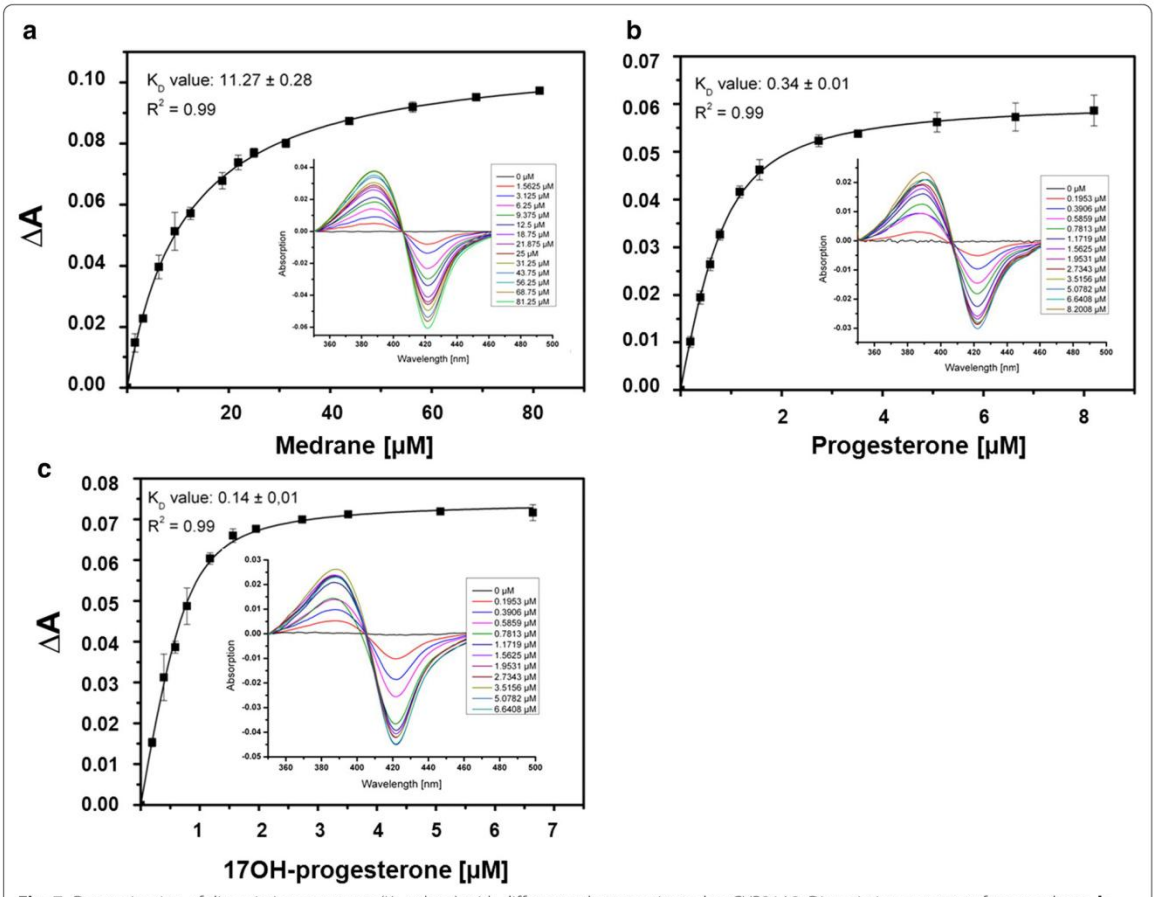

Fig. 7 Determination of dissociation constants (K_D values) with different substrates titrated to CYP21A2. Dissociation constants for **a** medrane, **b** progesterone and **c** 17OH-progesterone were determined by titration of bovine CYP21A2 with increasing concentrations of the particular substrate leading to a typical type I shift. Hyperbolic regression resulted in the respective K_D values, indicating the extent of the binding affinity towards the enzyme

dependent reactions. Therefore, we performed a scale-up of the most efficient whole-cell system consisting of bovine CYP21A2 and the heterologous redox partner proteins arh1 and etp1^{fd}. Protein expression was performed in Erlenmeyer flasks, and after a washing step the cell density for the biotransformation was adjusted to 72 g L^{-1}. The reaction took place in the bioreactor BiostatQ® with 500 mL resting cells in a defined buffer medium. 1000 mg medrane were added and the reaction was performed for 20 h with a stir velocity of 700 rpm. With this simple scale-up approach from a reaction volume of 25 mL in shaking flasks up to 500 mL in a bioreactor, a higher cell density was reached and a maximum product yield of 0.65 g premedrol per L d^{-1} could be achieved. Considering the reaction's time dependence it was shown that the initial rate of 88 mg L^{-1} h^{-1} within the first 3 h dropped to 15 mg L^{-1} h^{-1} within the last 15 h of the biotransformation (Fig. 8). The decrease of the reaction rate was already observed in shaking flasks and in other CYP dependent whole-cell systems indicating limiting factors for a continuous biotransformation [46]. In case of CYP21A2, protein stability as a limiting factor could be excluded by CO difference spectroscopy of samples taken before and after bioconversion, which showed a highly stable enzyme (Additional file 4: Fig. S4). Furthermore, we could confirm by Western blot analysis that there exists an optimal stoichiometry of CYP21A2 and the redox proteins arh1 and etp1^{fd}. Regarding a biotechnological application the next step would be the establishment of a controlled fermentation process ensuring a stable pH, carbon source as well as substrate feeding and, overall, a sufficient supply with oxygen needed for a CYP catalyzed reaction. Nevertheless, this scale-up approach implicates the potential to increase the whole-cell system's efficiency by a biotransformation in a bioreactor and already produced nearly gram amounts of product per liter and day (Additional file 5: Fig. S5, Additional file 6: Table S1).

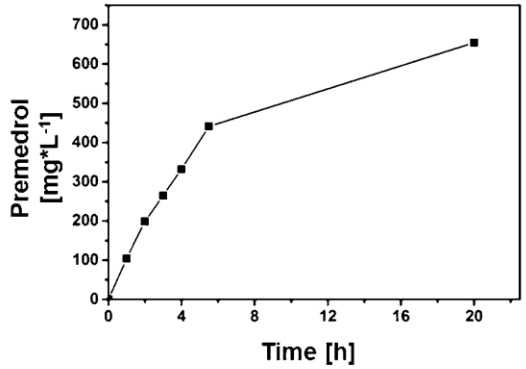

Fig. 8 Time-dependent premedrol formation by the CYP21A2 based whole-cell system with arh1 and etp1^{fd} in a bioreactor. Whole-cell biotransformation in a bioreactor was performed with cells of C43(DE3), transformed with the vector p21b_ArEt, which is encoding bovine CYP21A2 as well as arh1 and etp1^{fd}, by using resting cells in a volume of 500 mL and a cell density of 72 g L^{-1}. Samples were taken after certain time points and analyzed via HPLC to examine the temporal premedrol increase

Conclusions

In this work, we demonstrated that the mammalian CYP21A2, which is involved in mammalian steroid hormone biosynthesis and catalyzes a stereo- and regioselective 21-hydroxylation of progesterone and 17OH-progesterone, also exhibits a high potential as biocatalyst for medrol production. It could be demonstrated with purified enzyme as well as in a newly established *E. coli* whole-cell system that CYP21A2 is able to convert medrane to premedrol, an important precursor of medrol, via a selective oxofunctionalization at C21. Since a sufficient electron supply is an essential factor influencing CYP dependent reactions, the focus has shifted from the natural CYP21A2 redox partner, the membrane protein CPR, to alternative redox proteins. Therefore, five different redox systems were introduced and examined regarding velocity and endpoint yield. The systems containing the ferredoxin reductase arh1 from *S. pombe* were similar in their endpoint independent on the used ferredoxin. With regard of the initial reaction rate it is shown in Fig. 5, that the redox chain consisting of arh1 and its natural ferredoxin etp1^{fd} is two times faster than all other systems, possibly due to the fact that the electron transfer between a natural redox pair is much more effective and faster than in the system with Adx (Fig. 5; Table 2). Taken together, the use of soluble enzymes with a high expression yield seems to be the best choice for the system's stability and effectiveness, which is underlined by an ideal protein stoichiometry, confirmed by Western blot analysis of the three participating proteins (Fig. 6). Compared to the CPR based system the premedrol yield could be enhanced about the 3.6 fold by the use of other redox partners, which shows the high impact of a sufficient, stable and suitable electron supply during biotransformation. We could achieve 320 mg L^{-1} d^{-1} in shaking flasks experiments. A scale-up approach to 500 mL in a bioreactor, including an increase of the cell density, could further improve the overall yield up to a maximum of 0.65 g L^{-1} d^{-1} which shows the potential of the system for an industrial application and an important step towards the replacement of the chemical premedrol synthesis by a biocatalytic approach.

Methods

Chemicals, kits, enzymes and primary antibodies

All chemicals were from standard sources and of highest purity. Solvents used for chromatographic analysis were of gradient grade. Steroids for analysis and whole-cell biotransformation were from Sanofi, Frankfurt-Höchst (DE) and of highest purity. Restriction enzymes were obtained from New England Biolabs (Frankfurt, DE), kits for plasmid preparation and DNA purification from Machery-Nagel (Düren, DE) and the FastLink™ DNA Ligation Kit from Epicentre Biotechnologies (Madison, US). The primary antibody against arh1 was obtained from Charles River Laboratories (Sulzfeld, Germany), against etp1^{fd} from BioGenes (Berlin, Germany) and against bovine CYP21A2 from antikoerper-online.de (Aachen, Germany).

Bacterial strains and cultivation

Plasmid preparation and cloning experiments were carried out with *E. coli* TOP10 (Invitrogen, San Diego, CA, USA). Protein synthesis and whole-cell biotransformation were performed with *E. coli* strain C43(DE3) [47]. The cultivation took place in Luria–Bertani broth (BD, Heidelberg, DE) or in terrific broth (TB) complex medium. Transformed cells were stored as glycerol stock with a 1:1 mixture of an overnight culture and glycerol (50 %) at −80 °C.

Molecular cloning

Expression vector for bovine and human CYP21A2

The cDNAs for bovine and human *CYP21A2* was constructed according to Arase et al. with a replacement of the N-terminal hydrophobic anchor region with MAKK TSSKGK from CYP2C3 and a 6× Histidin tag for protein purification [24, 48]. It was digested with NdeI and BamHI and ligated into the pET17b expression vector (Novagen). The constructed vectors are subsequently designated as pET17b_21b and pET17b_21h.

Construction of vectors for a whole-cell biotransformation

All constructed vectors for a whole-cell biotransformation are based on the pET17b expression vector. All constructs consist of bi- or tricistronic transcription units with the *CYP21A2* cDNA sequence and one or two redox partner cDNAs downstream of it (Fig. 2, Additional file 1: Fig S1, Additional file 2: S2).

The vector pET17b_21b served as a backbone for the construction of the vector p21b_bRED, containing the cDNA for bovine *CYP21A2* and its natural redox partner, the bovine cytochrome P450 reductase (*CPR*), as a bicistronic transcription unit. A CPR containing vector was used for PCR amplification of *CPR* cDNA [49]. The forward primer contains a BamHI restriction side followed by a ribosomal binding side and the respective coding region. The appropriate reverse primer carries the C-terminal coding region including the stop codon and a NotI restriction side. The PCR product was digested and ligated between the BamHI and NotI sides of pET17b_21b, resulting in the bicistronic construct p21b_bRED. The vector p21h_bRED was cloned likewise.

The vector p21b_AdAx contains a tricistronic transcription unit, consisting of the cDNA for bovine *CYP21A2*, bovine adrenodoxin reductase (*AdR*) and truncated bovine adrenodoxin ($Adx_{1–108}$). Bovine AdR and Adx represent the mitochondrial redox system, which is proved to interact with CYP21A2. The vector Twin11B1 served as a backbone for the construction and carries the cDNA for human *CYP11B1*, bovine *AdR* and bovine *Adx* in a tricistronic arrangement [20]. Firstly, an undesired HindIII site had to be removed within the *CYP21A2* sequence by QuikChange® Site-Directed Mutagenesis. The resulting cDNA for bovine *CYP21A2* was amplified by PCR with pET17b_21b as a template. The forward primer is equal to the existing DNA sequence and contains an NdeI restriction site. The reverse primer carries the end of the coding region and a HindIII site. Both PCR product and the vector Twin11B1 were digested and the *CYP21A2* cDNA was ligated between the NdeI and HindIII sites of Twin 11B1 by a replacement of the *CYP11B1* sequence against the *CYP21A2* cDNA which results in the tricistronic vector p21b_AdAx.

The vector p21b_FrAx was constructed according to p21b_AdAx and contains the *E. coli* reductase Fpr instead of AdR, which is cloned through the HindIII and KpnI sites. Origin of the Fpr sequence was the vector pET_MR6 [27].

For cloning of the vectors harboring components of the redox system from *S. pombe* the vector pBar_Twin_pombe served as a template, carrying the cDNA for adrenodoxin reductase homologue 1 (*arh1*) and the ferredoxin domain of the electron transfer protein 1 (*etp1^{fd}*) [36, 38, 41]. In a first step, the AdR sequence of the vector p21b_AdAx was replaced by the *arh1* cDNA which was amplified via PCR using pBar_Twin_pombe as a template. The forward primer carried a HindIII restriction site as well as a following ribosomal binding site while the reverse primer was identical to the C-terminal sequence including a KpnI restriction site. The amplified PCR product was digested and cloned into the likewise digested p21b_AdAx. The resulting vector p21b_ArAx contains a tricistronic construct composed of the cDNAs for *CYP21A2*, *arh1* and *Adx*.

In the next step, the vector p21b_ArEt was constructed based on the backbone of the vector p21b_ArAx, which contains both components of the *S. pombe* redox system. Again, pBar_Twin_pombe served as a template for a PCR amplification of *etp1^{fd}*. The forward as well as the reverse primer were identical to the etp1^{fd} cDNA sequence, carrying a KpnI

and an EcoRI restriction site. The PCR product was digested and ligated between the KpnI and EcoRI restriction sites of the likewise digested p21b_ArAx resulting in an exchange of Adx by etp1^{fd}. The vector p21b_FrEt was cloned likewise. All used primers are listed in Additional file 6: Table S6.

Protein expression and purification

Expression and purification of electron transfer proteins

Bovine AdR and Adx as well as arh1, Fpr and etp1^{fd} were expressed in *E. coli* and purified as described before [27, 34, 36, 38, 50].

Bovine CPR was synthesized in *E. coli* and purified via Immobilized Metal Ion Affinity Chromatography (IMAC) as described elsewhere [49].

Expression and purification of bovine and human CYP21A2

C43(DE3) were co-transformed with the expression vector pET17b_21b and the vector pGro12 which carries the genes for the molecular *E. coli* chaperones GroEL/ES to ensure a proper protein folding and a correct integration of the heme cofactor. For the seed culture, 10 mL LB medium, supplemented with 100 µg mL^{-1} ampicillin for pET17b_21b selection and 50 µg mL^{-1} kanamycin for pGro12 selection, were inoculated with transformed cells from a glycerol stock and grown overnight at 37 °C with 160 rpm. For the main culture, 250 mL TB medium, supplemented with 100 µg mL^{-1} ampicillin and 50 µg mL^{-1} kanamycin, were inoculated with 1/100 (v/v) of the seed culture and grown at 37 °C with 190 rpm to an OD_{600} of 0.5. At this time point, gene expression was induced by adding 1 mM isopropylthiogalactopyranosid (IPTG), 1 mM δ-aminolevulinic acid as heme precursor and 4 mg mL^{-1} L-arabinose for the induction of the chaperones GroEL/ES. Protein synthesis was carried out at 27 °C with 150 rpm for 38 h. Cells were harvested at 4,000*g* for 20 min at 4 °C.

Cell pellets were diluted in lysis buffer, consisting of 50 mM potassium phosphate buffer (pH 7.4), 500 mM sodium acetate, 0.1 mM EDTA, 20 % glycerin, 1.5 % sodium cholate, 1.5 % Tween20, 0.1 mM PMSF and 0.1 mM DTE. Cells were disrupted by sonification and centrifuged with 30,000*g* at 4 °C for 30 min. The supernatant was taken for the subsequent purification. The 3 step protein purification by Immobilized Metal Ion Affinity Chromatography (IMAC) and DEAE Sepharose as well as SP Sepharose for ion exchange chromatography was done as previously described by Arase et al. [24].

UV/vis spectroscopy

CO difference spectroscopy of reduced CYP in complex with CO was carried out for a qualitative and quantitative enzyme characterization following the typical absorption maximum at 450 nm with an extinction coefficient of 91 mM^{-1} cm^{-1} [13].

Difference spectroscopy was performed to examine the binding behavior of CYP21A2 natural and unnatural CYP21A2 substrates as previously described by using tandem cuvettes. CYP21A2 was dissolved in buffer (50 mM potassium phosphate (pH 7.4), 20 % glycerol, 0.5 % sodium cholate and 0.05 % Tween 20) and titrated with increasing amounts of substrate in DMSO. Difference spectra were recorded from 350 to 500 nm. The values from three titrations were averaged and the K_D values were determined by fitting the plots with hyperbolic regression or tight binding quadratic equation with OriginPro 9.1G [51].

Reconstituted in vitro assays

The in vitro reconstitution assay was performed in a final volume of 250 µL with 50 mM HEPES buffer (pH 7.4) containing either 100 µM DLPC and 20 % glycerol for the CPR or 0.5 % Tween20 for all other redox proteins. The final concentration of CYP21A2 was 0.5 µM, the concentration of arh1 and AdR 0.5 µM, of Adx and etp1^{fd} 10 µM, of the Fpr 25 µM and of the CPR 1 µM, respectively. Additionally, the mixture contained a NADPH regeneration system consisting of 5 mM glucose-6-phosphate, 1 mM $MgCl_2$ as well as glucose-6-phosphate dehydrogenase. The particular steroid substrate was added in a concentration range of 100–400 µM. The reaction was started with 5 mM NADPH or NADH and incubated shaking for 30–40 min at 37 °C. The assay was stopped by addition of 250 µL chloroform, steroids were extracted twice with chloroform, dried and stored at −20 °C for HPLC analysis.

Whole-cell biotransformation with different redox systems in shaking flasks

Protein synthesis of bovine and human CYP21A2 and the respective redox partners for a whole-cell biotransformation was performed as described above by co-transformation of C43(DE3) cells with the particular bi-or tricistronic vector and the pGro12. After 28 h expression time, cells were harvested at 4,000*g* for 15 min at room temperature. The cell pellets were washed with 50 mM potassium phosphate buffer and cell wet weight was adjusted to 24 g L^{-1}. The whole-cell biotransformation was carried out with resting cells in 50 mM potassium phosphate buffer (pH 7.4) supplemented with 2 % glycerol, 1 mM IPTG, 1 mM δ-Ala, 4 mg mL^{-1} arabinose and 25 µg mL^{-1} polymyxin B. The reaction volume was 25 mL in 300 mL baffled Erlenmeyer flasks. The steroid substrate was solved in DMSO and added in concentrations ranging from 200 to 1.2 mM. The whole-cell reaction mixture was incubated at 27 °C with 145 rpm for 24 h. Samples for HPLC analysis were taken at different time points, extracted twice with chloroform, dried and stored at −20 °C.

Whole-cell biotransformation in a bioreactor

Protein synthesis of bovine CYP21A2 and the redox partners arh1 and etp1^{fd} for a whole-cell biotransformation was performed as described above, by co-transformation of C43(DE3) cells with the p21b_ArEt vector and the pGro12. After 28 h expression time, cells were harvested at 4,000*g* for 15 min at room temperature. The cell pellets were washed with 50 mM potassium phosphate buffer and cell wet weight was adjusted to 72 g L^{-1}. The whole-cell biotransformation was carried out with resting cells in 50 mM potassium phosphate buffer (pH 7.4) supplemented with 2 % glycerol, 1 mM IPTG, 1 mM δ-Ala, 4 mg mL^{-1} arabinose and 25 µg mL^{-1} polymyxin B. The reaction volume was 500 mL and the biotransformation was carried out in the bioreactor BiostatQ® with a stir velocity of 700 rpm at 27 °C. The steroid substrate was dissolved in DMSO and added in concentrations up to 1000 mg L^{-1}.

Steroid analysis via RP-HPLC

Steroid analysis was carried out by reversed-phase high performance liquid chromatography using a Jasco reversed phase HPLC system of the LC900 series and a 4.6 mm × 125 mm NucleoDur C18 Isis Reversed Phase column (Macherey–Nagel).

The reconstituted in vitro assays were analyzed with an acetonitril/water gradient at 240 nm within 15 min at 40 °C and a flow rate of 0.8 mL min^{-1}.

The whole-cell conversion was measured with an acetonitril/water gradient at 240 nm within 30 min at 40 °C and a flow rate of 0.8 mL min^{-1}.

Western blot analysis

Samples from the culture, co-expressing bovine CYP21A2, arh1 and etp1^{fd}, were taken, adjusted to OD 1 and centrifuged. The pellet was suspended in 100 µl SDS-PAGE loading buffer and boiled for 10 min. 6 µL of the sample in case of CYP21A2 and arh1 and 3 µL in case of etp1^{fd} were separated on a 12 % acrylamide gel according to Laemmli et al. [52]. For Western blot analysis, proteins were transferred to hybond-ECL nitrocellulose membranes (Amersham, GE Healthcare, England) [53]. The membranes were blocked overnight in 3 % milk powder in 30 mL TBS (50 mM Tris–Cl pH 7.5, 400 mM NaCl, 0.15 % Tween 20). After blocking, the membranes were washed three times for 15 min with TBS and afterwards incubated for 1.5 h with the respective primary antibody, dissolved 1:1000 in TBS. After three following washing steps with TBS, incubation with the secondary horseradish-linked goat antirabbit IgG antibody (Dako, Glostrup, Denmark), diluted 1:3000 in TBS, took place for 1.5 h. In the following step, the membranes were washed three times for 15 min with PBS (10 mM potassium phosphate buffer pH 7.4, 150 mM NaCl) and afterwards, the protein-antibody conjugates were visualized by addition of 4-chloro-1-naphthol (2 mL; 3 mg/mL in ethanol) in 25 mL PBS supplemented with 10 µL H_2O_2. Relative intensity of the protein bands was measured with Image Lab 3.0 from BioRad (München, Germany). The determination of the protein yield was performed by comparing the sample amount (Fig. 6a–c, lane 1) to increasing concentrations of purified protein, for etp1^{fd} 10, 20, 30, 40, 50, 75 and 100 ng (Fig. 6a, lanes 2–8), for arh1 25, 50, 75, 125, 187.5 and 250 ng (Fig. 6b, lanes 2–7) and for bovine CYP21A2 24, 48, 95, 143, 191 and 239 ng (Fig. 6c, lanes 2–7). The relative lane intensities, which correlate with the respective protein masses, were determined and compared to the intensity of the whole-cell system sample. Mass values were converted into the amount of substances and extrapolated to the expression yield per liter culture.

Additional files

Additional file 1: Fig. S1. HPLC chromatograms of the *in vivo* and *in vitro* conversion of medrane with human CYP21A2 and CPR. **a** Medrane was converted with resting cells of C43(DE3) containing the human CYP21A2 isoform and CPR encoding vector p21h_bRED. Samples were taken after 24 h and extracted for HPLC analysis. The steroids were separated by an acetonitril:water gradient. **b** Substrate conversions of medrane were performed with purified human CYP21A2 and its redox partner CPR for 30 min. Steroids were extracted and analyzed by HPLC to verify a selective conversion of medrane to premedrol, respectively. The steroids were separated by an acetonitril:water gradient, showing the 21-hydroxylated product premedrol.

Additional file 2: Fig. S2. Vector maps of constructed vectors for a CYP21A2 based whole-cell system in *E. coli* with Adx as final electron donor. Three vectors with tricistronic transcription units were constructed, based on the pET17b vector with an inducible T7 promoter and an ampicillin resistance gene, with Adx as final electron donor in combination with one of the reductases, AdR (p21b_AdAx), arh1 (p21b_ArAx) or Fpr (p21b_FrAx).

Additional file 3: Fig. S3. Vector maps of constructed vectors for a CYP21A2 based whole-cell system in *E. coli* with etp1^{fd} as final electron donor. Two vectors with tricistronic transcription units were constructed, based on the pET17b vector with an inducible T7 promotor and an ampicillin resistance gene, with etp1^{fd} as final electron donor in combination with the reductases arh1 (p21b_ArEt) or Fpr (p21b_FrEt).

Additional file 4: Fig. S4. *In vitro* conversion of medrane with the redox systems AdR/Adx/CYP21A2 or arh1/Adx/CYP21A2 with either NADH or NADPH. 400 µM Medrane was converted in a reconstituted *in vitro* assay with Adx based redox systems containing AdR or arh1 as reductase. To each system either NADH or NADPH was added to verify the ability of arh1 to receive electrons from NADH. AdR served as a negative control. All values represent the mean of triplicates with the respective standard deviation.

Additional file 5: Fig. S5. CO difference spectra of bovine CYP21A2 before and after 24 h biotransformation. Cells were harvested before and after 24 hours biotransformation. COD was performed with the respective lysate, each showing a typical absorption maximum at 450 nm in a reduced state in complex with CO which indicates a correct heme insertion. The solid line shows the COD before biotransformation, the dashed one after 24 h bioconversion in buffer.

Additional file 6: Table S1. Sequences of used primers with indication of their purpose. Restriction sites are in bold letters and base exchanges are signed in bold and cursive.

Abbreviations
CYP21A2: 21-hydroxylase; *E. coli*: *Escherichia coli*.

Authors' contributions
SA carried out the presented experiments and drafted the manuscript. LS participated in the establishment of the expression and purification of human CYP21A2. FH participated in the design of the study, interpretation of the results and manuscript drafting. BJ performed the biotransformation experiments in the bioreactor. RB participated in the design of experiments, the interpretation of the results and in manuscript drafting.

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Competing interests
The authors declare that they have no competing interests.

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2.2 Simone Brixius-Anderko et al. (2016)

An indole deficient *Escherichia coli* strain improves screening of cytochromes P450 for biotechnological applications

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An indole deficient *Escherichia coli* strain improves screening of cytochromes P450 for biotechnological applications

The impact of indole on cytochromes P450

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Abstract

Escherichia coli has developed into an attractive organism for heterologous cytochrome P450 production, but in some cases was restricted as host in view of a screening of orphan cytochromes P450 or mutant libraries in the context of molecular evolution due to the formation of the cytochrome P450 inhibitor indole by the enzyme tryptophanase (TnaA). To overcome this effect, we disrupted the *tnaA* gene locus of *E. coli* C43(DE3) and evaluated the new strain for whole-cell substrate conversions with three indole sensitive cytochromes P450, myxobacterial CYP264A1 and CYP109D1 as well as bovine steroidogenic CYP21A2. For purified CYP264A1 and CYP21A2, the half maximal inhibitory indole concentration was determined to be 500 µM and 140 µM, which is within the physiological concentration range occurring during cultivation of *E. coli* in complex medium. Biotransformations with C43(DE3)_Δ*tnaA* achieved a 30% higher product formation in case of CYP21A2 and an even 4 fold increase with CYP264A1 compared to C43(DE3) cells. In whole-cell conversion based on CYP109D1, which converts indole to indigo, we could successfully avoid this reaction. Results in microplate format are indicating that our newly designed strain is a suitable host for a fast and efficient screening of indole influenced cytochromes P450 in complex medium.

Keywords

Cytochrome P450, *E. coli*, gene disruption, indole, screening

Abbreviations

CYP21A2, 21-hydroxylase; *E. coli*, *Escherichia coli;* IPTG, Isopropyl-β-D-1-thiogalactopyranoside; OD, optical density

Introduction

The superfamily of cytochromes P450 (P450, CYP) represents a highly conserved group of external monooxygenases and can be found in all domains of life [1, 2]. The CYPs show a diverse substrate range and are able to introduce oxygen even into non-activated carbon bonds, which makes them very interesting for biotechnological applications [3]. However, since cytochromes P450 are external monooxygenases, they, in general, need an electron transfer system to allow oxygen activation and substrate conversion [4]. To date, the existence of various groups of redox systems, containing one or two components, or fused systems, which interact with the CYP via electrostatic interaction, has been described [5]. The number of cytochrome P450 complements (CYPomes) and respective redox transfer proteins is growing with every newly sequenced genome, revealing the impact of these enzymes on life in many aspects like environment, agriculture, human and animal health and biotechnology [6, 7]. Thus, it represents a big challenge to screen orphan CYPs to evaluate their biocatalytic potential as well as their substrate range. In particular, bacterial and fungal CYPomes offer a great biotechnological and pharmaceutical potential, since many of the orphan P450s showed new interesting reactions and are able to catalyze the conversion of diverse substrates [4]. Furthermore, the exploitation of bacterial CYPs as drug metabolizers is of great interest, since the demand of novel drug analogues and therapeutics is increasing. It has already been shown that CYPs of the actinomycetes *Streptomyces coelicolor* showed similar drug metabolizing properties as CYPs from the human liver, e.g. CYP2D6 or CYP3A4 [7, 8]. Inspired by these reports we have been engaged in studying the CYPome of the myxobacterium *Sorangium cellulosum* So ce56, which contains 21 CYP encoding genes [9]. Within our studies, we were also able to reveal the potential of some myxobacterial CYPs as drug metabolizers, especially for antidepressant, antipsychotic, and anticancer drugs, in which an *Escherichia coli* based whole-cell biocatalyst has been employed for the bioconversion [10, 11]. Aside from the screening of orphan CYPs, enzyme improvement by molecular evolution is of general interest whether to enhance poor turnover numbers, to find new non-natural reactions or to switch stereoselectivities [12-14]. For this purpose, a fast and reliable screening system in a suitable host is imperative. The bacterial host *E. coli* turned out to be highly suitable for the heterologous CYP expression and biotransformation because of the absence of intrinsic CYPs in this organism. To ensure optimal cell growth and sufficient protein expression for such CYP dependent biotransformation processes, a complex medium seems to be the best choice, but the liberation of metabolites during the bacterial growth, especially of indole, sometimes

interferes with the biotransformation process. The inhibitory effect of indole towards cytochrome P450 mediated reactions was firstly described by Girhard et al., who observed a decreased biotransformation in complex medium with whole cells of *E. coli* expressing CYP109B1 from *Bacillus subtilis* [15]. Furthermore, they proved on molecular level that the decrease of enzyme activity strongly depends on the concentration of indole. Investigating CYPs from the myxobacterium *Sorangium cellulosum* So ce56, Ringle et al. observed that the CYP264A1 mediated hydroxylation of 4-methyl-3-phenyl-coumarin to 4-hydroxymethyl-3-phenyl-coumarin was drastically decreased in complex medium [16]. The indole effect was verified by titration of increasing indole amounts to *E. coli* cultures grown in defined medium without tryptophan and it was found that the product formation decreased with increasing indole concentrations. Indole is generated from the degradation of the amino acid tryptophan by the enzyme tryptophanase, which catalyzes the reaction of tryptophan to indole, pyruvate and ammonium under glucose depletion [17, 18]. In recent years it was elucidated that indole is not only a simple side product of the amino acid metabolism but also acts as a signaling molecule for bacterial antibiotic resistance, biofilm formation and quorum sensing processes. Concerning *E. coli*, indole mediates pathogen resistance towards some species of the fungus *Candida i*n the human intestine [19-21]. The genome analysis showed that the tryptophanase gene locus is part of the tryptophanase operon comprised of three open reading frames, *tnaA, tnaB and tnaC*, in which *tnaA* encodes the tryptophanase and the protein product of *tnaB* acts as a low-affinity tryptophan transporter. The activity of both genes solely depends on the amount of tryptophan in the medium [22]. The third part of the operon, *tnaC*, encodes a small leader peptide, which participates in the regulation of the operon by an antitermination process. It has been observed before that the amount of tryptophan in a complex medium and the growth on glycerin leads to increased indole concentrations up to a millimolar range [22, 23], which is sufficient to inhibit the catalytic activity of the two CYPs investigated so far towards their sensitivity against indole. With regard to a screening of orphan P450s and their ability for a biotechnological application, an indole mediated inhibition, however, decreases the practicability and the reliability. Some strategies were developed to overcome the effect of indole on the CYP activity during biotransformation, e.g. growing the cells in a defined medium with low tryptophan amounts, which, however, often leads to a decreased expression yield and a slower growth [24]. In addition, the transfer of the cells to buffer for the bioconversion has been practiced, but it is not suitable for an application of recombinant *E. coli* cells as a fast screening system because of the additional working steps [25].

Furthermore, indole has been identified as a substrate for some liver CYPs, this way suggesting a competition with other substrates [26].

Therefore, in this study we tested the sensitivity of two bacterial and one mammalian CYP towards indole to find out how general the effect of indole on the CYP activity might be. Moreover, we developed a strategy to avoid indole inhibition by disrupting the *tnaA* gene locus of the *E. coli* expression host, which allows a suitable cultivation of the biocatalyst in complex medium without inhibiting the CYP enzyme activity. As representatives we choose the myxobacterial CYPs CYP264A1 and CYP109D1 as well as the bovine steroidogenic CYP21A2. We demonstrate that the new strain of *E. coli* is ideally suited for both, the characterization of orphan CYPs and the screening for random mutant libraries of indole sensitive enzymes.

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Material and methods

Chemicals, kits and enzymes

All chemicals were from standard sources and of highest purity available. Solvents used for chromatographic analysis were of gradient grade. Restriction enzymes and DNA polymerases were obtained from New England Biolabs (Frankfurt, DE), kits for plasmid preparation and DNA purification from Macherey-Nagel (Düren, DE).

Bacterial strains and cultivation

Gene disruption, gene expression and whole-cell biotransformation were performed with *E. coli* strain C43(DE3) [27]. The cultivation of liquid cultures was carried out in Luria-Bertani broth (BD, Heidelberg, DE) or in terrific broth (TB). Agar plates consisted of LB medium with 1% agar. Transformed cells were stored as glycerol stock with a 1:1 mixture of an overnight culture and glycerol (50%) at -80°C.

Gene disruption by homologous recombination

The *tnaA* knock-out was performed using the Quick and Easy *E. coli* Gene Deletion Kit from Gene Bridges (Heidelberg, DE).

PCR amplification of a tnaA knock-out cassette

For a homologous RED/ET mediated recombination in *E. coli*, which is based on the λ phage recombination system, a gene disruption cassette had to be generated containing flanking regions of the respective gene. The gene disruption cassette FRT-PGK-gb2-neo-FRT contains flanking flippase recognition target (FRT) sites, which mediate a flippase dependent removal of the kanamycin resistance gene after successful genomic integration, the eukaryotic promoter pPGK for kanamycin resistance in mammalian cells, the promotor gb2 for the antibiotic resistance in *E. coli* and, finally, a kanamycin resistance gene. Therefore, primers were designed, which possess a 50 bp homology region with the *tnaA* gene locus as well as a 23-25 bp region corresponding to the gene disruption cassette, which mediates the kanamycin resistance. 50 ng of the knock-out cassette FRT-PGK-gb2-neo-FRT was used as template

DNA for a polymerase chain reaction (PCR) with the primer *tnaA*_1 (5'- ATG GAA AAC TTT AAA CAT CTC CCT GAA CCG TTC CGC ATT CGT GTT ATT GAA ATT AAC CCT CAC TAA AGG GCG G -3'), which contains the 5´ homologue region to the *tnaA* gene, and the primer *tnaA*_2 (5'-TTA AAC TTC TTT CAG TTT TGC GGT GAA GTG ACG CAA TAC TTT TGG TTC GTT ACG ACT CAC TAT AGG GCT CG -3'), possessing the particular 3´ region of the gene locus. The PCR was done with Phusion DNA Polymerase according to established protocols. The success of the PCR was verified by agarose gel electrophoresis. To improve the probability of the homologous recombination, the previously synthesized linear DNA fragment was further enlarged about 50 bp upstream and downstream of the prior *tnaA*_1 and *tnaA*_2 primers by a subsequent PCR. The primers *tnaA*_P_G (5'-TGT AAT ATT CAC AGG GAT CAC TGT AAT TAA AAT AAA TGA AGG ATT ATG TAA TGG AAA ACT TTA AAC ATC TCC-3') and *tnaA*_P_H (5'-TGT AGG GTA AGA GAG TGG CTA ACA TCC TTA TAG CCA CTC TGT AGT ATT AAT TAA ACT TCT TTC AGT TTT GCG-3') carry each 22 bp of the previously used primers and 50 bp of the respective *tnaA* gene locus. As a template the product of the previously performed PCR was used and the PCR was confirmed by agarose gel electrophoresis, yielding a modified linear gene disruption cassette with a total size of 1,836 bp, containing a 100 bp long flanking homologous sequence to the *tnaA* gene locus at 5´ and 3´.

Red/ET mediated homologous recombination

The Red/ET mediated homologous recombination was performed according to the Quick and Easy *E. coli* Gene Deletion Kit from Gene Bridges (Heidelberg, DE). The flippase mediated cleavage of the disruption cassette through the flanking FRT sides was performed with the vector pE-FLP (Addgene, Cambridge UK) carrying a weak constitutive pE phage promotor.

Indole test

To confirm the homologous recombination and subsequent disruption of the tnaA gene locus, an indole assay was performed. Cells were cultivated in LB medium at 37°C overnight. After cultivation, the cultures were overlaid with Kovács reagent (15 mL isoamyl alcohol, 1 g p-dimethylaminobenzaldehyde, 5 mL HCl conc.). The organic upper phase of indole producing cultures showed a purple stain, while the tryptophanase deficient strain did not show this purple color.

TnaA knock-out verification on a genetic level

Genomic *E. coli* DNA was isolated from C43(DE3), C43(DE3)_Δ*tnaA*_*neo*$^{+}$ as well as from flippase treated C43(DE3)_Δ*tnaA* to confirm the insertion of the gene disruption cassette and the subsequent removal of the kanamycin resistance gene by PCR and agarose gel electrophoresis. 1.5 mL of an *E. coli* overnight culture was centrifuged at maximum speed for 1 min. The cell pellet was suspended in 600 µL lysis buffer (9.34 mL TE buffer, 600 µL of 10% SDS, 60 µL proteinase K (20 mg/L)) supplemented with RNase A and incubated at 37°C for 1 h. After incubation, an equal volume of a chloroform/phenol/isoamylalcohol mixture (25/24/1) was added and mixed until the phases were combined. After centrifugation at maximum speed, the upper aqueous phase was collected. The previous step was repeated, until the protein layer occurring between the phases totally disappeared. To remove phenol from the aqueous phase, chloroform was added in an equal amount, and a further centrifugation step was performed. 3 volumes of cold ethanol were added to precipitate the DNA, mixed and incubated for 30 min at -20°C. After centrifugation for 15 min at 4°C, the supernatant was discarded and the DNA pellet was washed with 70% ethanol. The ethanol was removed after a final centrifugation step, and the DNA pellet was dried for further use. The PCR for the *tnaA* knock-out verification was performed with Phusion DNA polymerase according to standard protocols with the primers *tnaA*_P_G and *tnaA*_P_H used for the amplification of the gene disruption cassette. The PCR products of all three strains were subsequently analyzed by agarose gel electrophoresis.

Protein expression and purification

Bovine CYP21A2 as well as myxobacterial CYP264A1 and CYP109D1 from *Sorangium cellulosum* So ce56 were produced and purified as described previously [9, 16, 28]. The redox transfer proteins adrenodoxin reductase (AdR) and a shortened version of adrenodoxin (Adx_{4-108}) from *Bos taurus* were purified as described elsewhere [29, 30].

P450 activity assay

The *in vitro* reconstitution assays were performed in a final volume of 250 µL with 50 mM HEPES buffer (pH 7.4) containing 0.5% Tween20. The final P450 concentration was 0.5 µM, the concentration of adrenodoxin reductase (AdR) 0.5 µM and of adrenodoxin (Adx) 10 µM. Additionally, the mixture contained a NADPH regeneration system consisting of 5 mM glucose-6-phosphate, 1 mM $MgCl_2$ as well as 4 U/mL glucose-6-phosphate dehydrogenase. The particular substrate was added in a concentration of 200 µM – 400 µM. Indole was added with increasing concentrations in the range of 100 - 10,000 µM to identify a possible inhibitory effect. The amount of solvent was equal in every reaction mixture to avoid solvent effects. The reaction was started with 5 mM NADPH and incubated for 25-40 min at 37°C. The reaction was stopped by addition of 250 µL chloroform, the substances were extracted twice with chloroform, dried and stored at -20°C for HPLC analysis.

Whole-cell biotransformation in microtiter scale

C43(DE3) as well as C43(DE3)_Δ*tnaA* cells were used to perform recombinant protein synthesis and biotransformation with bovine CYP21A2 as well as myxobacterial CYP264A1 and CYP109D1. For bioconversion with CYP21A2, the cells were transformed with the vector p21b_ArEt, carrying the cDNAs for bovine CYP21A2, arh1 (adrenodoxin reductase homologue) and $etp1^{fd}$ (electron transfer protein 1) from *Schizosaccharomyces pombe* and an ampicillin resistance gene, and the vector pGro12, encoding the *E. coli* chaperones GroEL/ES for a correct protein folding, which mediates a kanamycin resistance [28, 31-33]. In order to generate CYP264A1 and CYP109D1-dependent biocatalysts, cells were transformed with a pET17b vector (Novagen, Darmstadt, Germany), encoding the respective CYP and the ß-lactamase, and the vector pCDF_dFA, which carries the cDNAs for the NADPH-dependent *E. coli* reductase Fpr and the ferredoxin Adx and mediates a streptomycin resistance [11]. The seed cultures were grown in LB medium with the particular antibiotics. The main cultures consisted of 1 mL TB medium in a 96 well plate (2.2 mL), supplemented with the respective antibiotics, 1 mM IPTG, 1 mM δ-aminolevulinic acid and 4 mg/mL arabinose in case of a GroEL/ES coexpression. Recombinant protein production was performed at 320 rpm at 27°C. After 24 h, the biotransformation was initiated by addition of the particular substrate and 25 µg/mL polymyxin B to enhance the substrate uptake into the bacterial cells. Samples were

taken after 24 h, extracted twice with chloroform, dried and stored at -20°C for HPLC analysis.

Substance analysis via reversed phase high performance liquid chromatography (RP-HPLC)

Steroid analysis was carried out by RP-HPLC using a Jasco reversed phase HPLC system of the LC900 series and a 4.6 mm × 125 mm NucleoDur C18 Isis Reversed Phase column (Macherey-Nagel).

The reconstituted *in vitro* assays were analyzed at 240 nm for steroid substrates and at 280 nm for 4-methyl-3-phenyl-coumarin within 15 min at 40 °C and a flow rate of 0.8 mL/min with the gradient: 100% solvent A (50% acetonitrile in water) for 8 min, 90% solvent B (100% acetonitrile) for 2 min and 100% solvent A for 5 min.

The *in vivo* experiments were measured within 30 min at 240 nm for steroid substrates and at 280 nm for 4-methyl-3-phenyl-coumarin and a flow rate of 0.8 mL/min with the gradient: 80% solvent A (10% acetonitrile in water) for 13 min, 60% solvent A for 7 min, 80% solvent B (100% acetonitrile) for 2 min and 80% solvent A for 8 min.

Indigo extraction and UV/vis spectroscopy

Myxobacterial CYP109D1 is able to hydroxylate indole to indoxyl, which subsequently dimerizes to the blue stain indigo. To verify the indigo formation, cells of C43(DE3) as well as C43(DE3)_Δ*tnaA*, both expressing CYP109D1 and the redox partners Fpr and Adx_{1-108}, were centrifuged at 4,000*g* for 15 min. The cells were washed with DMSO to solve the accumulated indigo and centrifuged. The indigo containing supernatant was spectroscopically analyzed by measuring a spectrum from 400 to 800 nm to verify indigo, which exhibits an absorption maximum at 620 nm [34].

CO difference spectroscopy of reduced CYP in complex with CO was carried out for a qualitative and quantitative enzyme characterization following the typical absorption maximum at 450 nm in a reduced state in complex with CO with an extinction coefficient of 91 mM^{-1} cm^{-1} [35].

Difference spectroscopy was performed using tandem cuvettes as previously described to examine the influence of substrate binding on the heme iron spin state equilibrium of the respective CYP and to determine dissociation constants (K_D). 3 µM myxobacterial CYP264A1 and CYP109D1 were dissolved in 10 mM potassium phosphate (pH 7.4) and titrated with increasing amounts of substrate dissolved in DMSO. Difference spectra were recorded from 350 to 500 nm. The peak-to-trough absorbance differences from three titrations were averaged and plotted against the substrate concentration. K_D values were determined by fitting a hyperbolic regression line to the data points with OriginPro 9.1G.

Results

The effect of indole on cytochromes P450 on a molecular level

In former studies it was demonstrated that indole, formed by *E. coli* in complex media, can inhibit some CYPs [15, 16]. On the other hand, indole was also shown to be a substrate of some mammalian P450s acting as a competitive inhibitor of the conversion of other substrates [26]. Therefore, we first wanted to study these effects in more detail by investigating three representative CYPs, two of bacterial origin as well as a microsomal steroid hydroxylase, to find out whether the interference of indole with these enzymes is more common than thought so far and could impede a screening in context of molecular evolution or the elucidation of orphan CYPs in general.

Indole mediated CYP inhibition

We have demonstrated before that indole inhibits the activity of recombinant CYP264A1 when added to *E. coli* grown in defined medium [16]. To investigate this effect in more detail on a molecular level, we used here purified CYP264A1 in a reconstituted *in vitro* system and studied the effect of indole on the conversion of 4-methyl-3-phenyl-coumarin.

First we examined the molecular interaction of indole with CYP264A1. For this, dissociation constants for indole and the substrate 4-methyl-3-phenyl-coumarin were determined. As shown in Figure 1, both, the substrate as well as indole, induced a type I spectrum, described by an increase of the absorption at 390 nm and a decrease at 420 nm. The titration of purified CYP264A1 with increasing amounts of the ligands resulted in a dissociation constant of 27 ± 4 µM for the substrate 4-methyl-3-phenyl-coumarin and 1.1 ± 0.1 mM for indole, suggesting a 40 times tighter binding of the substrate compared with indole (Figure 1 A, B). The calculated heme-percentage shift induced by indole for CYP264A1 was 20%. In order to determine the indole effect on the CYP mediated hydroxylation, CYP264A1 and the redox partners AdR and Adx were applied in an *in vit*ro activity assay. We chose AdR and Adx, as it turned out to be the best electron transfer system within *in vitro* assays for many of the myxobacterial CYPs [11, 16, 36]. Indole was added to the reaction mixture in a concentration ranging from 100 µM to 10 mM. The product formation was analyzed by HPLC and the total product yield was calculated. As a control, a reaction without addition of indole was carried out and the corresponding product amount was set to 100%.

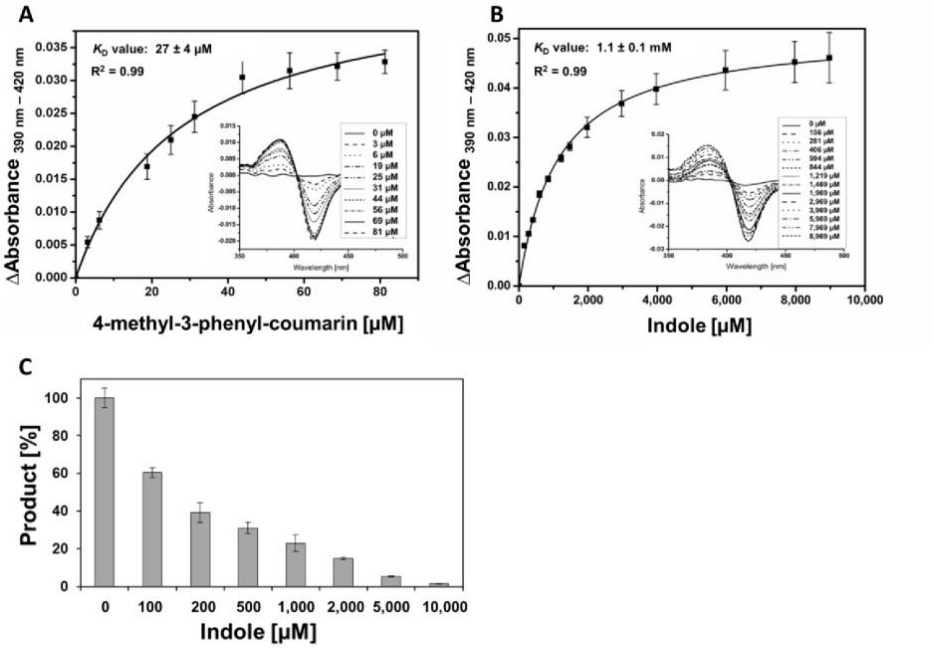

Figure 1: Effect of indole on CYP264A1 on a molecular level. The dissociation constants for **A.** 4-methyl-3-phenyl-coumarin and **B.** indole were determined by titration of CYP264A1 with increasing concentrations of the particular compound, leading to a type I shift (inset). K_D values were determined by fitting a hyperbolic regression line to the data points with OriginPro 9.1G, indicating the extent of the binding affinity towards the enzyme. **C** The relative percentage of the product formation in the presence of increasing concentrations of indole is shown. Indole was added with increasing concentrations in the range of 100 - 10,000 µM to identify an inhibitory effect. The values represent the mean of three independent experiments with the corresponding standard deviation.

A strong inhibitory influence of indole on the catalytic ability of CYP264A1 has been observed, in which the formation of the product 4-hydroxymethyl-3-phenyl-coumarin was decreased to 60% at an indole concentration of 100 µM (Figure 1 C), suggesting a strong effect at even low indole concentrations. At an indole concentration of 500 µM the yield of 4-hydroxymethyl-3-phenyl-coumarin decreased to 30% (Figure 1 C). The indole concentrations used here correspond well to those that have been observed during the cultivation of *E. coli* in

complex medium, in which up to 5 mM indole was detected [22]. At 5 mM only 3% of CYP264A1 activity remained, while it was nearly abolished at 10 mM. The determined half maximal inhibitory concentration (IC_{50}) was estimated as 141 µM.

Since the inhibitory effect of indole on a bacterial CYP was clearly demonstrated, we next aimed to examine its impact on a mammalian CYP, bovine CYP21A2, to show that an indole mediated inhibition is independent on the CYP origin. Recently, a new process for the biotechnological production of premedrol, the precursor of the pharmaceutically highly relevant synthetic glucocorticoid medrol (methylprednisolone) by using whole cells of *E. coli* expressing bovine CYP21A2 was published [28]. Since mammalian CYPs usually exhibit low turnover numbers, a molecular evolution of CYP21A2 could lead to an increased substrate conversion and facilitate an application of this enzyme in an industrial process, replacing the established chemical synthesis. For this, a suitable fast screening system has to ensure both, a sufficient protein expression and an unhindered biotransformation. Time consuming procedures like transferring the cells into buffer should be avoided.

Since even high concentrations of indole neither induce a type I nor a for nitrogen containing inhibitors typical type II shift during difference spectroscopy, we examined the effect of indole during medrane conversion in reconstituted *in vitro* assays employing bovine CYP21A2 as well as AdR and Adx as redox partners. The mitochondrial redox system has been used here, since it was shown to be very effective in *in vitro* assays with purified CYP21A2 [37]. As shown in Figure 2, the addition of 100 µM indole decreased the formation of the product premedrol to 80%, while 500 µM indole was determined to be the half maximal inhibitory concentration, demonstrating a the strong influence of indole also on the CYP21A2-mediated hydroxylation reaction.

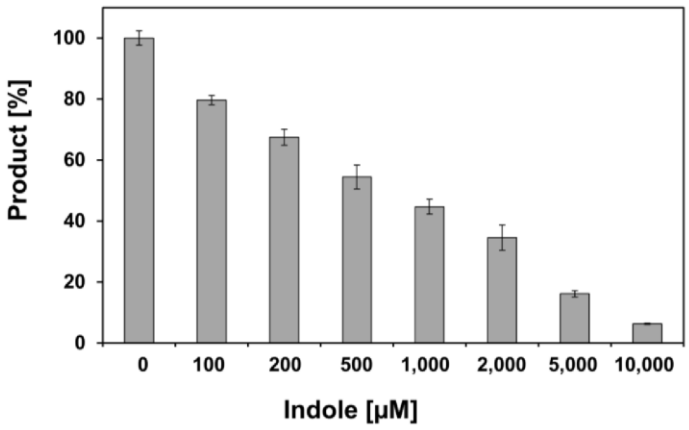

Figure 2: Inhibitory effect of indole on CYP21A2 activity on a molecular level. Indole was added in increasing concentrations in the range of 100 - 10,000 µM to verify an inhibitory effect during conversion of 400 µM medrane in *in vitro* assays. All values represent the mean of three independent trials with the respective standard deviation.

Indole converting CYPs

In addition to the observed effect as an inhibitor of CYPs, indole also can act as substrate and this way as competitive inhibitor of some CYPs. Gillam et al. showed the hydroxylation of indole to indoxyl, which subsequently dimerizes to the blue stain indigo by mammalian P450s [26]. When studying the biotechnological potential of the *S. cellulosum* CYPome, we also observed the formation of a blue precipitate during biotransformation in complex medium when using CYP109D1. The blue substance was spectroscopically identified as indigo, which is formed after the CYP109D1 mediated indole hydroxylation to indoxyl (3-hydroxyindole) [34] (Figure 3 A, B). The natural substrates of CYP109D1 are still unknown, however, the enzyme was able to convert fatty acids and norisoprenoids, such as α-ionone and β-ionone [9, 36]. Since in the whole-cell system indole was identified as an additional substrate for CYP109D1, we investigated its binding to CYP109D1 on a molecular level and observed a clear type I shift. The dissociation constant for indole was determined to be 162 ± 11 µM (Figure 3 C), indicating that it can act as a competitive inhibitor of the substrate conversion

catalyzed by CYP109D1. The calculated heme-percentage shift induced by indole for CYP109D1 was 20%.

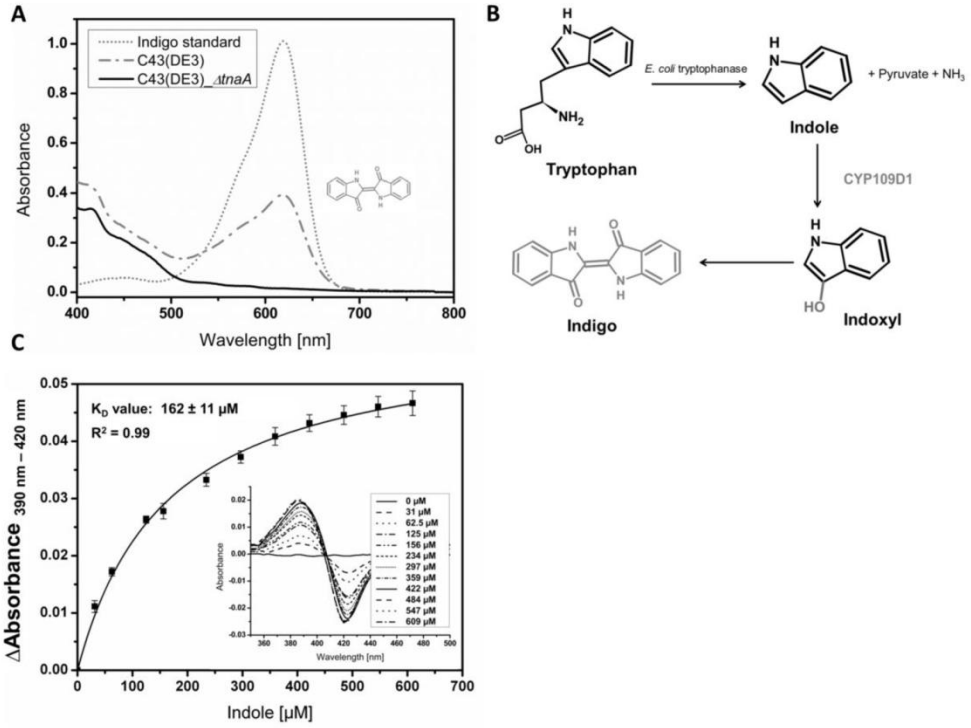

Figure 3: Indigo formation by CYP109D1 expressing cultures. A Indigo was extracted with DMSO from CYP109D1 and redox proteins expressing cultures of C43(DE3) cells (dashed/dotted line) as a consequence of indole hydroxylation. In agreement with the indigo standard (dotted line), the extracted indigo showed a typical absorption maximum at 620 nm. CYP109D1 expressing C43(DE3)_Δ*tnaA* cells did not show indigo accumulation (solid line). **B** A proposed schematic representation of the conversion of indole by CYP109D1 is shown. Tryptophan is converted by the *E. coli* tryptophanase to pyruvate, ammonium and indole. Indole serves as substrate for myxobacterial CYP109D1 and is hydroxylated to indoxyl, which subsequently dimerizes to indigo. **C** The dissociation constant of indole was determined by titration of CYP109D1 with increasing indole concentrations resulting in a typical type I shift (inset).

Generation of the tryptophanase deficient strain C43(DE3)_Δ*tnaA*

Since indole was demonstrated to be a quite general inhibitor during CYP dependent biotransformations, we attempted to abolish its function to avoid labor intensive processes like the change of culture media to buffer-systems or the removal of tryptophan from culture media. For this, we aimed to disrupt the tryptophanase gene locus (*tnaA*) in the genome of the *E. coli* strain C43(DE3), which should abolish the indole effect during bioconversion, which is also required for a fast and efficient substrate screening system in a multi-well format.

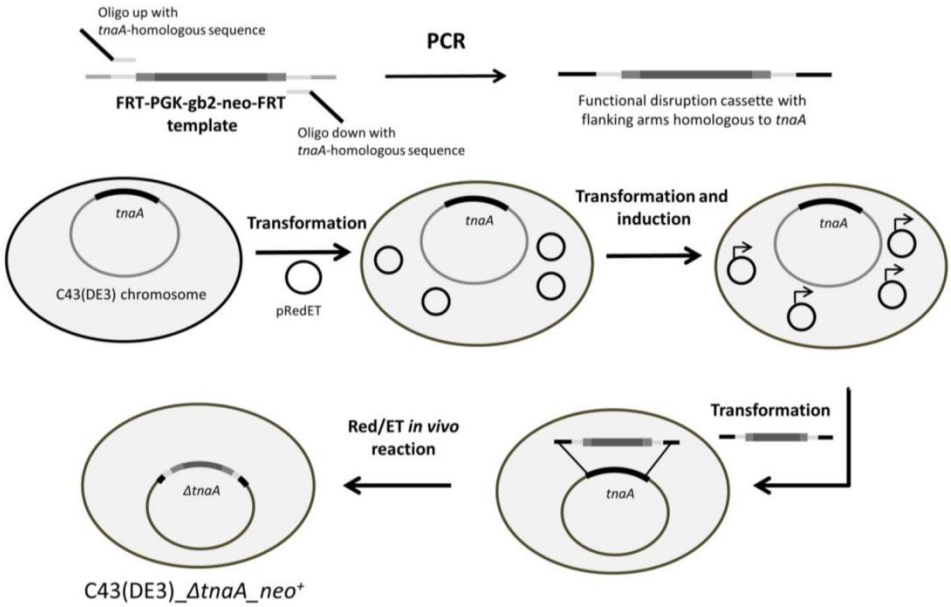

Figure 4: Generation of a *tnaA* gene knock-out strain. The schematic overview shows the generation of the C43(DE3)_Δ*tnaA* strain by homologous recombination. The linear disruption cassette FRT-PGK-gb2-neo-FRT was amplified by PCR with primers containing flanking regions homologous to the *tnaA* gene locus. C43(DE3) was transformed with the vector pRedET carrying the λ phage genes required for recombination and subsequently induced. After protein synthesis, the cells were transformed with the linear disruption cassette for homologous recombination resulting in the strain C43(DE3)_Δ*tnaA_neo*$^{+}$. (FRT = flippase recognition target sites; pPGK = eukaryotic promoter for kanamycin resistance in mammalian cells; gb2 = promotor for the antibiotic resistance in *E. coli*; neo = kanamycin resistance gene).

The gene disruption cassette FRT-PGK-gb2-neo-FRT, carrying 100 bp flanking regions of the *tnaA* gene, was created by polymerase chain reaction for the RED/ET mediated homologous recombination, and was verified by agarose gel electrophoresis. The amplified linear disruption cassette was consecutively introduced into C43(DE3) cells, which are expressing the required proteins for the recombination event, encoded by the pRed_ET vector (Figure 4). The bacterial cells containing a successfully integrated disruption cassette were subsequently selected on kanamycin agar plates and further cultivated in LB medium to verify the *tnaA* knock-out by an indole assay with Kovács reagent. The cultures were overlaid with Kovács reagent and the organic supernatant of indole positive cultures develops a purple color, while indole negative ones remain colorless. This way, we could easily select indole negative clones resulting in the strain C43(DE3)_Δ*tnaA*_*neo*$^{+}$, which was further transformed with the vector pE_FLP, consecutively expressing a flippase for a removal of the kanamycin resistance gene within the integrated disruption cassette by cleavage at the flanking FRT sites. To verify the successful gene disruption as well as the removal of the kanamycin resistance gene on a genetic level, we isolated genomic DNA from C43(DE3), C43(DE3)_Δ*tnaA*_*neo*$^{+}$ as well as from flippase treated C43(DE3)_Δ*tnaA* cells and a PCR was performed using primers *tnaA*_P_G and *tnaA*_P_H for the amplification of the gene disruption cassette. The agarose gel electrophoresis revealed a successful integration of the gene disruption cassette (1,836 bp) as well as the subsequent removal of the kanamycin resistance gene (317 bp) (Figure 5 A). Finally, after removal of the resistance gene, the constructed Δ*tnaA* strain as well as the origin strain C43(DE3) were tested with Kovács reagent, whereby the knock-out strain showed no indole formation at all.

To prove the influence of the gene knock-out on cell growth, samples were taken periodically from cultures grown in TB medium and the optical density (OD) was monitored at 600 nm. We observed that the cell growth of both strains is identical in the lag phase as well as in the exponential phase (Figure 5 B). From the beginning of the stationary phase, however, the C43(DE3)_Δ*tnaA* cells grow slightly slower than the C43(DE3) cells. At the end of the sampling after 25 h, the C43(DE3) cultures reached an OD_{600} of 7.7, while the knock-out cells reached 6.7.

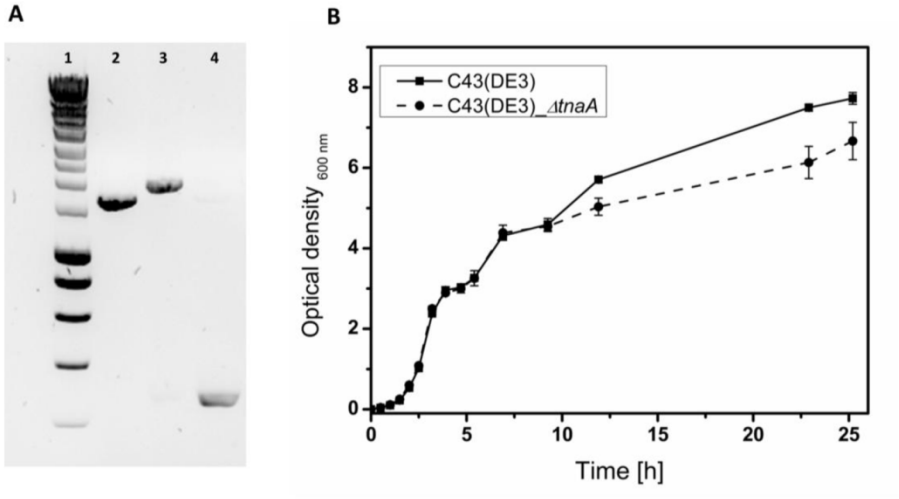

Figure 5: Characterization of a *tnaA* gene knock-out strain. A. PCR analysis of genomic DNA prepared from C43(DE3) shows a DNA fragment of 1,416 bp corresponding to a functional *tnaA* gene locus after agarose gel electrophoresis (lane 2). C43(DE3)_Δ*tnaA*_*neo*$^{+}$ cells containing the complete disruption gene cassette show a PCR product of 1,836 bp (lane 3). After a flippase treatment for the removal of the kanamycin resistance gene, a fragment of 317 bp occurs (lane 4). For analysis, the DNA marker smart ladder was used (lane 1). **B.** Growth curve of C43(DE3)_Δ*tnaA* and C43(DE3). The optical density at 600 nm of C43(DE3)_Δ*tnaA* (dashed line) and C43(DE3) (solid line) cultures was monitored. Samples were taken at several time points and the OD was determined by photometry. All values represent the mean of three independent cultures with the respective standard deviation.

CYP-mediated biotransformation using C43(DE3)_Δ*tnaA* knock-out cells

To evaluate the applicability of the created knock-out strain C43(DE3)_Δ*tnaA* for screening assays and biotransformations in microtiter plates, we compared its ability of substrate conversion after expression of various CYPs with that of the indole producing strain.

For this, we first examined the beneficial effect of the knock-out strain during biotransformation with the indole converting CYP109D1. Whole-cell biotransformation in a microplate scale was carried out in complex medium in the host C43(DE3) as well as C43(DE3)_Δ*tnaA* harboring CYP109D1 and the redox proteins Fpr and Adx. After extraction of the indole producing C43(DE3) cultures with DMSO after 48 h of cultivation, we verified the formation of indigo by spectral analysis, since the solution showed a typical absorption maximum at 620 nm (Figure 3 A, dashed/dotted line). As expected, spectral analysis of cultures with C43(DE3)_Δ*tnaA* showed no indigo formation due to a total depletion of indole (Figure 3 A, solid line). Thus, the tryptophanase knock-out was clearly shown to abolish the CYP109D1-dependent formation of indigo.

To evaluate the effect of the tryptophanase knock-out on the inhibition of CYP-dependent substrate conversion, whole-cell biotransformation in a 1 mL scale was carried out in complex medium in the host C43(DE3) as well as C43(DE3)_Δ*tnaA* harboring CYP264A1 as a representative of the myxobacterial CYPs and the redox proteins Fpr and Adx. Whole-cell biotransformation was performed with increasing amounts of the substrate 4-methyl-3-phenyl-coumarin to verify, whether the tryptophanase deficient strain is a convenient host for a sufficient biotransformation already with low substrate concentrations in microtiter scale. It was observed, that the substrate conversion with C43(DE3) showed a strong indole formation resulting a poor product yield (Figure 6 A), whereas the Δ*tnaA* disrupted strain was indole deficient and showed a significantly increased product formation (Figure 6 B). While using the C43(DE3)_Δ*tnaA* cells, 90% of 50 µM substrate was hydroxylated, and the product yield was further increased with increasing substrate concentration. However, only 35% of 50 µM substrate was converted with C43(DE3) cells, and even with higher substrate concentrations there was no increment in the product yield (Figure 6 C). An examination of the protein level showed that the C43(DE3)_Δ*tnaA* cells produces more enzyme with 236 ± 29 nmol/L expression culture than the C43(DE3) strain with 62 ± 5 nmol/L culture. Taken together, the

indole deficient strain achieves 3-4 fold higher product formation compared with the C43(DE3) cells in microtiter plates.

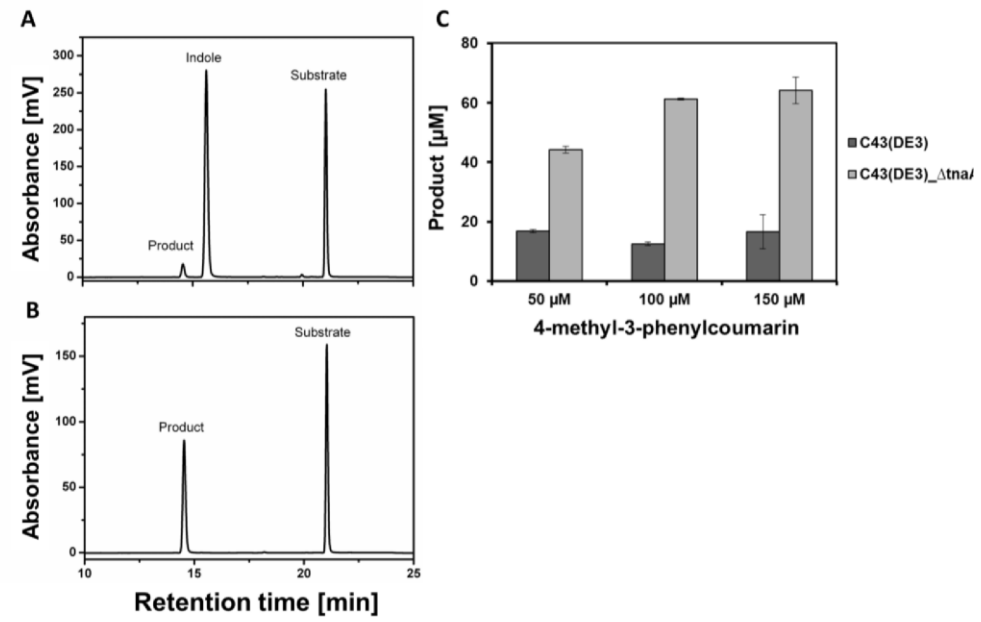

Figure 6: Whole-cell biotransformation with CYP264A1. Increasing concentrations of the substrate 4-methyl-3-phenyl-coumarin were converted either using C43(DE3) or C43(DE3)_Δ*tnaA* cells, harboring CYP264A1 and the redox transfer proteins Fpr and Adx in 1 mL complex medium for 24 h. Samples were extracted twice with chloroform and analyzed by HPLC. The HPLC chromatogram of the conversion with C43(DE3) shows a high indole formation, whereas it was lacking in the knock-out strain (**A**). Instead, the product yield increased significantly, when using the C43(DE3)_Δ*tnaA* strain (**B**). Even at low substrate concentrations, the indole deficient strain shows a superior product yield (**C**). All values represent the mean of three independent trials with the respective standard deviation.

Since the gene knock-out was demonstrated to be very beneficial for the substrate conversion using a bacterial CYP, we also investigated its effect on the whole-cell substrate conversion when using the mammalian CYP21A2. The formation of indole was only detected upon cultivation of the C43(DE3) strain, whereas no indole was found in the tryptophanase-deficient CYP21A2 expressing strain (Figure 7 A, B). The C43(DE3)_Δ*tnaA* strain showed ~ 30% higher premedrol formation compared with C43(DE3), independent on the used substrate concentration (Figure 7 C). Spectral analysis showed a 60% higher protein

production by the C43(DE3)_Δ*tnaA* cells (243 ± 71 nmol/L expression culture) than by the C43(DE3) strain (150 ± 37 nmol/L culture). This data successfully demonstrate that a tryptophanase deficient *E. coli* host has also beneficial effects during biotransformation with a mammalian CYP in a miniaturized screening approach.

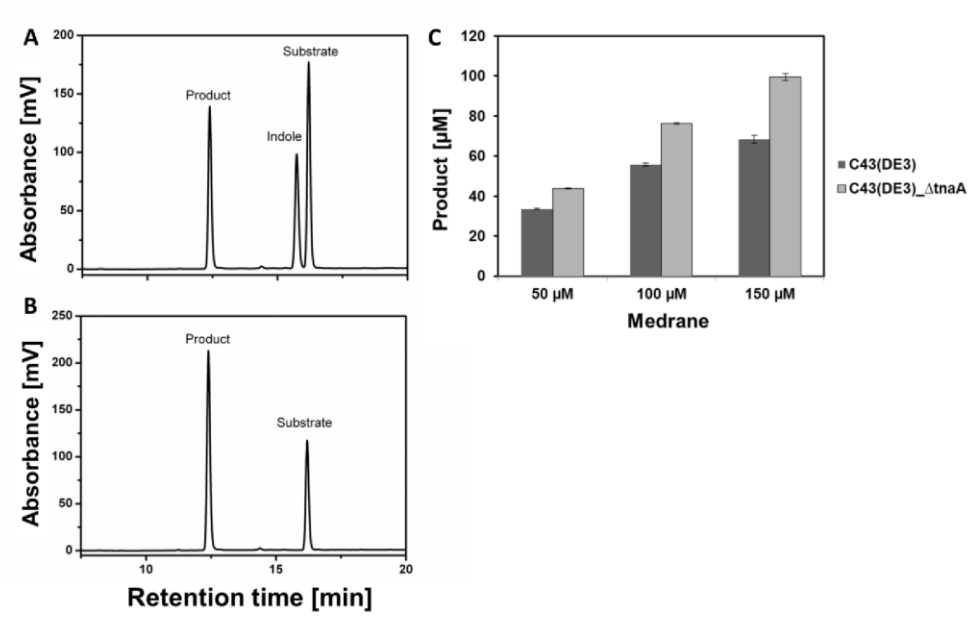

Figure 7: Whole-cell biotransformation with CYP21A2. Whole-cell substrate conversion of increasing concentrations of medrane with CYP21A2 and the redox transfer proteins arh1 and etp1^{fd} took place in 1 mL complex medium for 24 h. Samples were taken, extracted twice with chloroform and analyzed via HPLC. The HPLC chromatogram of the conversion with C43(DE3) shows a significant indole formation as well as the product premedrol (**A**). Biotransformation with the tryptophanase deficient strain lacks indole formation and reveals an improved depletion of the substrate medrane (**B**). Even at low substrate concentrations, the indole deficient strain shows a higher product yield (**C**). All values represent the mean of three independent trials with the respective standard deviation.

Discussion

In view of a possible biotechnological application, the characterization of orphan CYPs as well as P450 enzyme improvement by molecular evolution require suitable screening systems, which ensure appropriate cell densities, optimal heterologous protein expression and reaction conditions for an efficient substrate binding and conversion [7]. Though *Escherichia coli* is a popular and convenient expression host, even for mammalian membrane-bound CYPs, bioconversion in complex medium can be hindered by the accumulation of the aromatic compound indole, which acts as an inhibitor or substrate for CYP mediated catalysis [15, 26]. Though an inhibitory effect of indole on the myxobacterial CYP264A1 has already been observed by Ringle et al. during a whole-cell biotransformation process, the kind of inhibition has never been studied in depth on a molecular level. Hence, we chose this enzyme as a representative to examine its interaction with indole. Titration of CYP264A1 with indole showed a clear type I shift, which is typical for P450 substrates, although indole is not converted by CYP264A1. This observation is supported by data obtained with CYP109B1 from *Bacillus subtilis*, showing a clear type I shift, but no conversion [15]. The calculated dissociation constant of indole is in a millimolar range and 40 times higher than for the here used substrate 4-methyl-3-phenyl-coumarin. For a further examination, we performed *in vitro* assays with the CYP264A1 substrate 4-methyl-3-phenyl-coumarin and increasing amounts of indole. Despite of the high dissociation constant, the half maximal inhibitory concentration was determined to be about 140 µM - ten times lower than the K_D value. The data clearly show that indole interacts with the P450 active site, though the inhibitory effect of indole does, however, not directly correlate with its binding constant. A similar observation was made when using CYP109B1 from *B. subtilis.* The K_D value for indole was determined to be 24.5 µM and the dissociation constant for the investigated substrate was about 50 fold higher*;* nevertheless the *in vitro* assays only showed a decrease of 20% of total product formation after the addition of 100 µM indole [15]. Hence, the influence of indole is as individual as the enzyme to be considered. Furthermore, in view of the discrepancy between the determined dissociation constants and the indole-mediated inhibition in *in vitro* assays, its effect cannot be solely explained by the binding of indole to the active site, but also suggests the possibility of other kinds of interaction like allosteric ones or a disturbance of the CYP interaction with its redox partners, which is additionally underlined by the calculated heme-percentage shift of only 20%.

Since not only the elucidation of orphan CYPs is of increasing interest, but also an enzyme improvement for biocatalytic applications of CYPs, which naturally exhibit low turnover numbers, it is necessary to establish efficient screening systems, which ensure both recombinant protein synthesis and unhindered substrate conversion (Hannemann et al. 2007; Schiffer et al. 2015). Hence, we chose the mammalian steroidogenic CYP21A2 as a representative, which, as we have recently shown, catalyzes the 21-hydroxylation of medrane to premedrol, a precursor of the pharmaceutically interesting glucocorticoid methylprednisolone (medrol) [28]. Since medrane exhibits low binding to CYP21A2, it is crucial to prevent an indole mediated inhibition to be able to set up a sensitive and reproducible screening system for improved CYP21A2 mutants. Therefore, we examined the indole influence on the molecular level by titration of CYP21A2 with increasing amounts of indole. No effect on the spin state equilibrium could, however, be observed. Nevertheless, the half maximal inhibitory concentration during *in vitro* assays with purified enzymes turned out to be about 500 µM, which represents the physiological indole concentration, which ranges between 500 – 600 µM during our experiments. Thus, also the inhibition of mammalian CYPs by indole, as shown here for the microsomal steroid hydroxylase CYP21A2, has been clearly demonstrated.

To overcome indole dependent inhibition of CYPs without the renunciation of an efficient cultivation in complex medium in a screening setup, we performed a disruption of the *tnaA* gene locus, which encodes tryptophanase, a protein responsible for indole formation from the amino acid tryptophan. We chose the *E. coli* strain C43(DE3), which is a descendant of BL21(DE3) and highly suitable for the heterologous expression of sensitive proteins. Three mutations within the RNA polymerase *lac*UV5 promoter (P_{lacUV5}) result in a lower transcription rate of the gene of interest, which minimizes the uncoupling of transcription and translation and leads to a more stable protein expression [27, 38, 39]. In addition, this strain develops intracellular membranes, which are supposed to stabilize heterologously expressed proteins and to avoid inclusion bodies [40]. We successfully disrupted the *tnaA* gene locus by homologous recombination, which was examined on genomic level by PCR analysis, and proved the absence of indole with a colorimetric assay using Kovács reagent and HPLC analysis. The growth behavior of the developed strain C43(DE3)_Δ*tnaA* was subsequently evaluated by monitoring the optical density over time. The growth during the lag and the exponential phase was comparable to that of the wild type, whereas we observed a slightly slower cell density during the stationary phase. In the context of other studies, it was also observed that a *tnaA* disruption strain of *E. coli* JM101 accumulates less biomass than the

wild type. Furthermore, this strain showed acetate and lactate formation, which was explained by an altered carbohydrate metabolism due to a lack of indole, since indole is supposed to act as a signaling molecule under carbohydrate depletion during batch cultivation to ensure a sufficient nutrition of the culture [23].

To verify the beneficial effect of the constructed C43(DE3)_*ΔtnaA* strain, we performed whole-cell biotransformation experiments with the above-mentioned myxobacterial and mammalian CYPs. In case of myxobacterial CYP264A1, the *in vitro* results are confirmed by whole-cell biotransformation with the knock-out strain, resulting in a three to four fold higher product formation compared with C43(DE3), even at low substrate concentrations (Figure 6). Ringle et al. reported indole experiments where they added indole to C43(DE3), cultivated in a defined medium without tryptophan, to examine the inhibitory effect during biotransformation [16]. Here, the half maximal inhibitor concentration was determined to be around 400 µM, which correlates with the physiological concentration during cultivation in complex medium and underlines our results. Considering a screening of orphan CYPs, like the myxobacterial ones from *S. cellulosum* So ce56, a sufficient substrate conversion is imperative to find new substrates or to elucidate natural ones. The developed tryptophanase deletion strain thus is a convenient alternative for an application in the screening for new substrates. In addition, in case of CYP21A2-mediated biotransformation, the beneficial effect of the C43(DE3)_*ΔtnaA* strain is also observed, whereby a 30% higher product formation could be achieved compared with the C43(DE3) strain. This considerably improves the sensitivity for the screening of a mutant library in context of molecular evolution of this industrially relevant enzyme. Additionally, a higher protein yield could be achieved with the indole deficient cells compared to the C43(DE3) strain suggesting a beneficial impact on protein biosynthesis by indole absence, which also contributes to enhancing the product formation. Indole is supposed to act as a signaling molecule under carbohydrate depletion during batch cultivation to ensure a sufficient nutrition of the culture. Mainly, it acts as activator of the regulatory protein Crl, which subsequently increases the activity of the RpoS subunit of RNA polymerase. RpoS regulates the transition between exponential and stationary phase by inducing the expression of genes, which are altering carbohydrate metabolism ensuring cell survival. In absence of indole, the metabolism is not directed to carbohydrate and energy scavenging, which could be beneficial for maintaining gene expression [41, 42]. In view of this data it is not clear whether the beneficial effect of the C43(DE3)_*ΔtnaA* in biotransformation assays comes from indole depletion and therefore an exclusion of

inhibitory effects or from a higher protein expression level, which clearly is a consequence of the *tnaA* knock out.

Generally, it is known that cultivation in microtiter plates results in a decrease in mass transfer and oxygen supply, which are complicating conditions during a CYP-mediated biotransformation [43, 44]. Hence, indole inhibiting effects represent additional limitations under microtiter conditions, which emphasizes the high suitability of our indole deficient C43(DE3) strain in context of the screening of biotechnological relevant orphan CYPs as well as of mutant libraries of well characterized CYPs.

Moreover, the deletion strain is beneficial, when substrate conversion has to be studied with CYPs, which use indole as a substrate and this way inhibit the conversion of the substrate of interest. Using CYP109D1 it was shown that the knock-out of the tryptophanase completely avoids indigo formation from indole catalyzed by this CYP. Since an indole converting enzyme could mislead the product pattern during a screening and also impedes the product purification for a subsequent identification, our knock-out strain is highly suitable to verify a clear product formation, only originating from the substrate to be tested. Aside the inhibitory effect on CYP-mediated biotransformation, an absence of indole comprises additional advantages. It is known that indole serves as signaling molecule and induces the expression of xenobiotic transport systems, which lead to a decrease of the sensitivity towards antibiotics resulting from a permanent secretion [20, 45]. Since the heterologous expression of proteins mostly comes from vector systems, which carry an antibiotics resistance gene for a strict selection of vector carrying colonies, plasmid maintenance might be ensured by indole depletion.

In summary, the *tnaA* gene disruption in *E. coli* represents a sophisticated solution to avoid indole-mediated inhibition of heterologously expressed CYPs and to verify an enhanced product formation as well as a clear product pattern. Especially in view of an elucidation of orphan CYPs or of mutant library in context of molecular evolution we showed by representative bacterial and mammalian CYPs that our indole deficient strain strongly facilitates screening procedures in a microtiter scale and contributes to a possible biotechnological application of these versatile proteins.

Conflict of interest

The authors declare that they have no competing interests.

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2.3 Lina Schiffer et al. (2015a)

A recombinant CYP11B1 dependent *Escherichia coli* biocatalyst for selective cortisol production and optimization towards a preparative scale

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MICROBIAL CELL FACTORIES

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A recombinant CYP11B1 dependent *Escherichia coli* biocatalyst for selective cortisol production and optimization towards a preparative scale

Lina Schiffer, Simone Anderko, Anna Hobler, Frank Hannemann, Norio Kagawa and Rita Bernhardt*

Abstract

Background: Human mitochondrial CYP11B1 catalyzes a one-step regio- and stereoselective 11β-hydroxylation of 11-deoxycortisol yielding cortisol which constitutes not only the major human stress hormone but also represents a commercially relevant therapeutic drug due to its anti-inflammatory and immunosuppressive properties. Moreover, it is an important intermediate in the industrial production of synthetic pharmaceutical glucocorticoids. CYP11B1 thus offers a great potential for biotechnological application in large-scale synthesis of cortisol. Because of its nature as external monooxygenase, CYP11B1-dependent steroid hydroxylation requires reducing equivalents which are provided from NADPH via a redox chain, consisting of adrenodoxin reductase (AdR) and adrenodoxin (Adx).

Results: We established an *Escherichia coli* based whole-cell system for selective cortisol production from 11-deoxycortisol by recombinant co-expression of the demanded 3 proteins. For the subsequent optimization of the whole-cell activity 3 different approaches were pursued: Firstly, *CYP11B1* expression was enhanced 3.3-fold to 257 nmol∗L^{-1} by site-directed mutagenesis of position 23 from glycine to arginine, which was accompanied by a 2.6-fold increase in cortisol yield. Secondly, the electron transfer chain was engineered in a quantitative manner by introducing additional copies of the *Adx* cDNA in order to enhance *Adx* expression on transcriptional level. In the presence of 2 and 3 copies the initial linear conversion rate was greatly accelerated and the final product concentration was improved 1.4-fold. Thirdly, we developed a screening system for directed evolution of CYP11B1 towards higher hydroxylation activity. A culture down-scale to microtiter plates was performed and a robot-assisted, fluorescence-based conversion assay was applied for the selection of more efficient mutants from a random library.

Conclusions: Under optimized conditions a maximum productivity of 0.84 g cortisol∗L^{-1}∗d^{-1} was achieved, which clearly shows the potential of the developed system for application in the pharmaceutical industry.

Keywords: Cortisol, Human CYP11B1, Steroid biotransformation, Whole-cell biocatalysis, *E. coli*

Background

Cortisol, the major human glucocorticoid, plays a crucial role in the physiological adaption to stress, the regulation of energy mobilization and immune response [1]. Its anti-inflammatory and immunosuppressive effects render it a powerful agent for the abatement of classical inflammatory symptoms like pain or swelling that occur in the course of acute and chronic inflammatory or autoimmune diseases. Moreover, cortisol serves as an intermediate in the production of synthetic glucocorticoids, which can exhibit even greater glucocorticoid effects but less mineralocorticoid side effects. Prednisolone, for example, is derived from cortisol by a microbial 1,2-dehydrogenation [2]. The hydroxyl group in position 11β of the cortisol molecule and its synthetic derivatives is the key functionalization that provides its glucocorticoid effects. It is the same functionalization that is the most difficult one to be introduced chemically or microbially in the preparative synthesis of cortisol. In current industrial production it is carried out as the final step of a hemi synthesis by microbial transformation of 11-deoxycortisol with fungal cultures of the genus *Curvularia* in a scale of about 100 tons per year [3] by taking advantage of the organism's endogenous

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steroid 11β-hydroxylase activity [4]. However, this process suffers from poor selectivity. Purification and characterization of the responsible enzyme revealed low regioselectivity as 11β-hydroxylation of the substrate is accompanied by 14α-hydroxylation [5,6]. Consecutively, it is of great interest to develop alternative biocatalysts for a more selective and efficient introduction of the 11β-hydroxyl group into synthetic glucocorticoids.

In the human adrenal cortex, which represents the principal tissue for the biosynthesis and secretion of gluco- and mineralocorticoids, cortisol is formed selectively from 11-deoxycortisol by the 11β-hydroxylase CYP11B1 (human steroid 11β-hydroxylase) (Figure 1) [7-10]. Hence, in the context of cortisol production CYP11B1 also constitutes an attractive candidate for a biotechnological application.

CYP11B1 belongs to the evolutionary highly conserved superfamily of cytochromes P450 (P450). P450s catalyze versatile biotransformations of a wide range of substrates in all domains of life. It is mainly their capability to activate molecular oxygen and to incorporate one oxygen atom into a substrate molecule leading to a regio- and stereoselective hydroxylation that vests them a tremendous biotechnological potential in the synthesis of pharmaceuticals and fine chemicals [11-14]. Due to their nature as external monooxygenases, P450s require an external electron donor, which is in general NAD(P)H, and an electron delivering system composed of one or more additional proteins [15]. In case of CYP11B1, which represents a mitochondrial P450, the respective electron transfer chain is constituted of AdR, an NADPH-dependent flavoprotein, and Adx, a [2Fe-2S]-cluster protein that interacts with the P450. Such complexity of P450 systems along with the necessity of a costly cofactor is so far one of the determining factors that restrict the employment of P450 catalysts in a larger scale. The most promising approach to overcome these limitations is the employment of whole-cell systems that offer cofactors from their metabolism and a cellular environment for the support of protein stability and do not require time-consuming purification steps [12,16]. The current state of molecular biology and recombinant protein expression enables the exploitation of different microbial hosts for application of biotechnologically interesting enzymes. CYP11B1 could already be applied in engineered yeast strains (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) that convert 11-deoxycortisol to cortisol [17-19] or even accept simple carbon sources as substrate when additional sterol providing and modifying genes are engineered and introduced [20]. However, optimization of these systems towards a relevant scale is a great challenge. Our laboratory previously reported the first expression of *CYP11B1* in a bacterial host (*Escherichia coli*, *E. coli*) for purification and enzymatic characterization [21]. Subsequently, we decided to use this fast-growing and genetically amenable microorganism, which does not possess any endogenous, by-product generating P450s, and established the first bacterial whole-cell system for application of CYP11B1 in cortisol preparative scale biosynthesis. The entire redox chain consisting of AdR, Adx and the P450 was introduced into *E.coli*. For optimization, CYP11B1 expression was enhanced by site-directed mutagenesis, the co-expression of Adx was quantitatively adjusted on transcriptional level and CYP11B1 was engineered by molecular evolution towards higher activity.

Results

Establishment of a CYP11B1 based whole-cell system for cortisol synthesis in *E. coli*

In order to employ human CYP11B1 for steroid hydroxylation in *E. coli*, we created the plasmid Twin_11B1 which is based on the pET-17b vector and carries the cDNAs of human CYP11B1 including the modifications described in the Material and methods section, bovine AdR and bovine Adx in a tricistronic transcription unit separated by ribosomal binding sites. This enables the reconstitution of a functional P450 system in the host organism. The *E. coli* strain C43(DE3), which has previously been reported as advantageous for the synthesis of membrane proteins [22], was co-transformed with the new plasmid and the chaperone encoding plasmid pGro12 [23]. Chaperone synthesis supports the proper

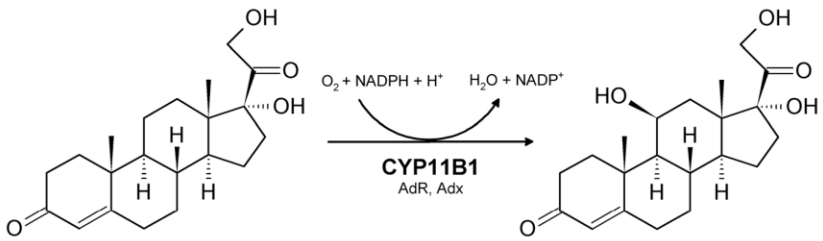

Figure 1 Scheme of the CYP11B1-catalyzed 11β-hydroxylation of 11-deoxycortisol yielding cortisol. Electrons are transferred from NADPH via AdR and Adx to CYP11B1, which activates molecular oxygen and incorporates one oxygen atom into the steroid substrate by means of a hydroxylation in a regio- and stereoselective manner, while the other oxygen is reduced to water.

folding of membrane proteins in the prokaryotic host [21,24-26]. Protein production was carried out in a complex medium and could be confirmed by Western Blot analysis with primary antibodies raised against CYP11B1, AdR and Adx, respectively.

Subsequent transformation of 11-deoxycortisol was conducted with non-growing cells in buffer supplemented with glycerol as carbon source to ensure a sufficient availability and regeneration of NADPH for the P450 reaction [27]. Thereby, a fixed cell density of 25 g_{wcw}/L was adjusted in all experiments. HPLC analysis of extracts from the resting cells demonstrates a selective CYP11B1 dependent 11β-hydroxylation of 11-deoxycortisol yielding cortisol (Figure 2). Steroids were identified via their retention times in comparison with standards from commercial sources.

As the solubilty of steroidal compounds can be a limiting factor for their bioconversion [12], we subsequently evaluated the effect of different dissolving agents for the addition of 11-deoxycortisol on the activity of the new whole-cell system. Each agent was added to a final concentration of 6% (vol/vol). While the employment of ethanol lead to the lowest cortisol yield and cyclodextrines and a 1:1 mixture of EtOH and PEG-400 slightly improved the final yield, the best results were obtained with DMSO, which was consecutively used for substrate supply in all subsequent experiments.

Optimization of CYP11B1 expression

In order to improve the *CYP11B1* expression in *E. coli* and thus the activity of the recombinant system, we performed site-directed mutagenesis in position 23 of the *E. coli* adapted sequence. Glycine, which corresponds to the published wildtype amino acid in that position [28,29], was replaced by the hydrophilic amino acid arginine. An analogous replacement from glycine to arginine, which was introduced into human *CYP19* when performing N-terminal replacements with related sequences from other P450s by Kagawa et al. [30], was reported to significantly enhance the expression in *E. coli.* The corresponding residue in human CYP11B1 was identified by primary sequence alignment. The expression level of the 2 CYP11B1 variants was estimated by CO-difference spectroscopy (Figure 3). The introduction of the arginine residue could succesfully enhance CYP11B1 level from 79 to 257 nmol∗L^{-1} and no significant reduction over the conversion period could be observed.

Cortisol formation by both enzyme variants in the whole-cell system was monitored in a time-dependent manner. In general, the system exhibited a linear volumetric productivity in an initial phase of at least 12 h. Afterwards the velocity of cortisol formation decreased and a final product concentration was reached after 30 h. The deployment of CYP11B1 G23R for the whole-cell conversion of 11-deoxycortisol increased the final cortisol yield after 30 h by a factor of 2.6 compared with the G23 variant from 239 to 631 mg∗L^{-1} (Figure 4). The initial linear productivity was enhanced in the same range from 11 to 27 mg∗L^{-1}∗h^{-1}. Therefore, CYP11B1 G23R is applied for all further experiments and will be

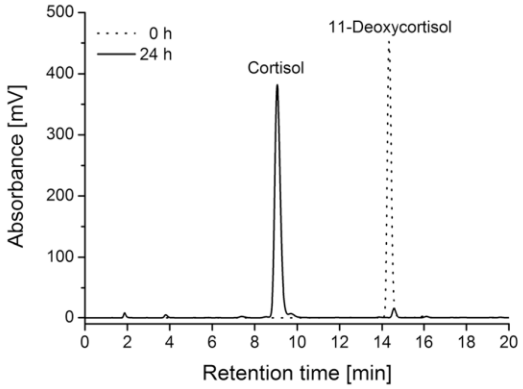

Figure 2 HPLC chromatogram of the CYP11B1 dependent conversion of 11-deoxycortisol to cortisol by recombinant *E. coli*. Chromatograms were obtained from extracted samples of *E. coli* cultures after 0 h (dotted line) and 24 h (solid line) of incubation after transfer into potassium phosphate buffer and addition of 1.2 mM substrate. Substance peaks obtained with an acetonitrile/water gradient represent cortisol with a retention time of 9.0 min and 11-deoxycortisol with a retention time of 14.3 min.

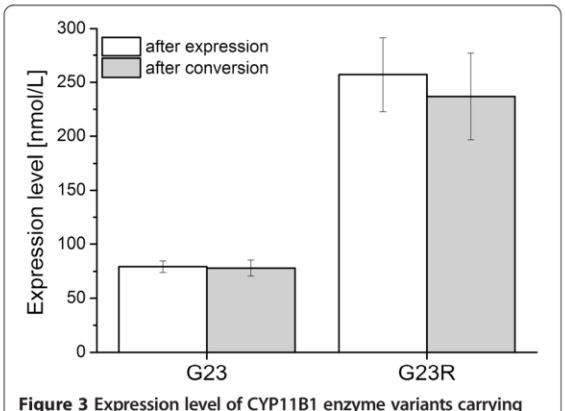

Figure 3 Expression level of CYP11B1 enzyme variants carrying either glycine (G23) or arginine (G23R) in position 23. Recombinant *E. coli* cultures expressing either wild type or mutant were harvested after the expression period (white bars) or at the end of substrate conversion (grey bars) under the conditions described in Material and methods. P450 concentration was determined by CO-difference spectroscopy with the supernatant of the cell lysate after ultracentrifugation. Values represent the mean of three experiments with the respective standard deviation.

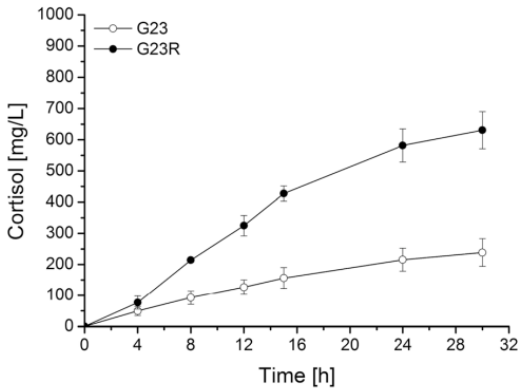

Figure 4 Comparison of the whole-cell cortisol formation by CYP11B1 enzyme variants carrying either glycine (G23) or arginine (G23R) in position 23 in recombinant *E. coli*. Reactions were performed after a 21-h expression period in TB medium with resting cells in the presence of 6% DMSO and 3 mM 11-deoxycortisol. Extracted steroids were analyzed by RP-HPLC. Values represent the mean of three conversion experiments conducted in parallel with respective standard deviation.

termed Pa1 (Parent generation 1) in the following molecular evolution studies.

Influence of Adx copy number

As electron supply frequently constitutes the limiting step in the efficiency of P450 systems [31-33], our next approach for improving the whole-cell activity of CYP11B1 was an increase of the amount of Adx in the system, in order to enhance electron transfer to CYP11B1. For that, we constructed variants of the expression plasmid Twin_11B1 with up to 4 copies of the Adx cDNA by successively integrating additional copies including a 5′-ribosomal binding site at the end of the trancription unit according to Blachinsky et al. [34]. The relative increase of *Adx* expression was estimated by Western Blot (Figure 5) and evaluation of the Adx signal with an imaging software. With the introduction of a second cDNA copy *Adx* expression was increased approximately 2.4-fold and a maximum of *Adx* expression (3.3-fold increase) was reached with the insertion of a third copy which could not be further augmented by a fourth copy. No influence on *CYP11B1* expression was observed by CO-difference spectroscopy, when 2 or 3 copies of *Adx* were present on the expression vector. With the insertion of a fourth *Adx* copy, the CYP11B1 titer was, however, reduced by 50% to approximately 120 nmol∗L^{-1} and the construct was thus not further investigated.

Subsequent whole-cell conversions conducted with 2 and 3 *Adx* copies increased the final product concentration from 631 mg∗L^{-1} for the initial system with 1 Adx to 877 and 828 mg∗L^{-1}, respectively (Figure 6).

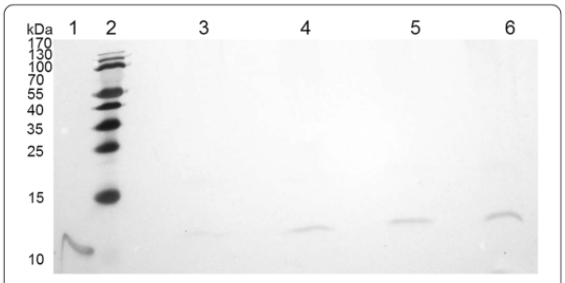

Figure 5 Western blot analysis of Adx expression by recombinant *E. coli* cells. *Lane 1* shows purified bovine Adx_{4-108} (~11,8 kDa). *lane 2* shows a pre-stained protein marker with molecular weights indicated on the left of the picture. *Lanes 3* to *6* represent the lysate of *E. coli* cells expressing the CYP11B1 redox system from variants of the Twin_11B1 plasmid carrying 1 to 4 copies of bovine Adx_{1-108} (~12,0 kDa). Adx detection was carried out with polyclonal antibodies and a peroxidase-based color reaction. The relative expression level of Adx was determined by measuring the band intensity with the Image Lab software.

Although both plasmids enabled a comparable final yield, the presence of 2 Adx copies also greatly enhanced the initial productivity over the first 12 h from 27 to 52 mg∗L^{-1}∗h^{-1}, while a third copy diminished this rate again to 37 mg∗L^{-1}∗h^{-1} but exhibited a longer phase of linearity in time-dependent product formation leading to a comparable final yield.

Development of a screening system for CYP11B1 activity

For further improvement of the CYP11B1 activity using molecular evolution, we adapted the biotransformation

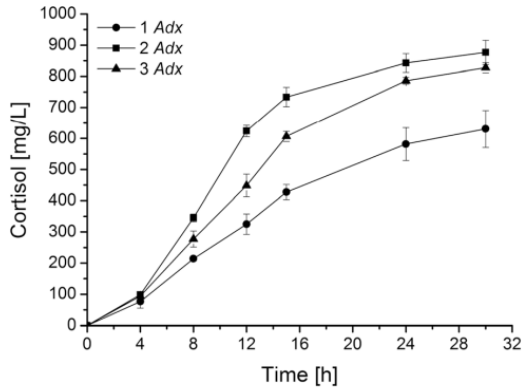

Figure 6 Comparison of the cortisol formation by recombinant *E. coli* transformed with different versions of the P450 system encoding plasmid Twin_11B1 with 1 (circles), 2 (squares) or 3 (triangles) copies of the Adx cDNA. Reactions were performed after a 21-h expression period in TB medium with resting cells in the presence of 6% DMSO and 3 mM 11-deoxycortisol. Extracted steroids were analyzed by RP-HPLC. Values represent the mean of three conversion experiments conducted in parallel with respective standard deviation.

with the *E. coli* whole-cell system and its subsequent evaluation to a microtiter plate format which enables a high and robot-assisted throughput. Culture size was scaled down to 1 mL and substrate conversion was carried out in TB medium with inoculation, induction of protein expression and addition of the substrate 11-deoxycortisol at the same time in order to reduce working steps to a minimum. For evaluation of enzyme activity, we employed a fluorescence assay, which makes use of the fluorescence developed by steroids with an intensity in dependence on the substitution of the steran scaffold [35-37]. As cortisol exposes a higher fluorescence than 11-deoxycortisol due to the additional hydroxyl group that is introduced in position 11β, enzyme variants with an increased hydroxylation activity can easily be selected. In order to ensure optimal conditions for the detection of mutants with improved activity, conversion with the parental enzyme variant Pa1 (CYP11B1 G23R) was tested with different substrate concentrations and the fluorescence was determined after 48 h of incubation in comparison with control cultures that were incubated with the respective 11-deoxycortisol concentration but without induction of protein expression. A clear difference between unspecific and steroid specific fluorescence could be observed as well as a significant increase of the relative fluorescence by a factor of more than 3 upon induction of protein expression, which proofs the presence of an active P450 system and thus the applicability of the system for monitoring 11-deoxycortisol conversion to cortisol (Figure 7).

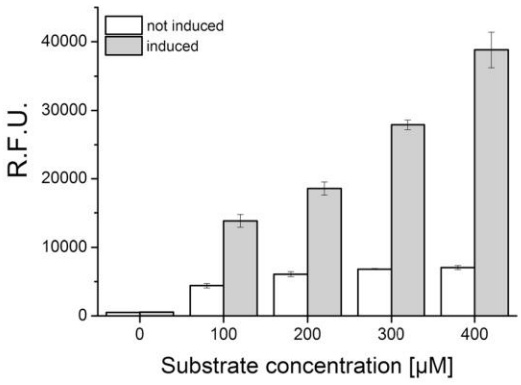

Figure 7 Relative fluorescence developed by recombinant *E. coli* cultures after incubation with different 11-deoxycortisol concentrations. Cells were transformed with Twin_11B1 and pGro12 and incubated with 11-deoxycortisol for 48 h in TB medium containing 1 mM δ-Ala (white bars) that has been additionally supplemented with 1 mM IPTG and 4 mg/mL arabinose for induction (grey bars). Steroid specific fluorescence was generated by the described assay and measured in relative units (R.F.U.) at λ_{ex} 485 nm and λ_{em} 535 nm. Values represent the mean of triplicates with standard deviations.

Higher substrate concentrations result in an increased activity, which is displayed by an increased fluorescence signal and which was confirmed by HPLC analysis. Steroids were identified by their retention time and relative quantification depicted 20.2 ± 2.9% conversion of 11-deoxycortisol to cortisol at a concentration of 400 μM, which was subsequently chosen for the selection of activity improved mutants.

Generation and screening of a CYP11B1 mutant library

A random CYP11B1 mutant library was created by epPCR applying the sequence of CYP11B1 Pa1 as a template. The emerged sequence variants were cloned into the Twin plasmid and C43(DE3) *E. coli* cells were co-transformed with the mutant Twin_11B1 plasmid library as well as pGro12 and were spread on agar plates. Sequencing of 10 randomly picked clones revealed a mutational frequency of 2.83 base exchanges per kilobase. Approximately 1000 clones were screened with the fluorescence assay for enhanced hydroxylation activity towards 11-deoxycortisol. From these, 53 clones, which exhibited an at least 1.5-fold higher fluorescence signal than the CYP11B1 Pa1 control incubated on the same plate, were re-screened in triplicates. For 3 mutants, which still showed an average increase of the fluorescence signal by more than the 1.5-fold, an activity increase between the 1.7- and 2.4-fold under screening conditions could be confirmed by HPLC. Sequencing identified the amino acid replacements H171L, Q166R/L271M and S168R/M286I/Q315E, respectively, for the selected clones. For the identification of the residues which cause the increased activity in case of the double and triple mutant all observed exchanges were introduced separately into CYP11B1 Pa1 by site-directed mutagenesis and analyzed for their whole-cell activity in microtiter plates using HPLC. Their activities regarding cortisol formation in comparison with the CYP11B1 Pa1 enzyme are shown in Figure 8.

The amino acid exchange Q166R, which occurred in the double mutant Q166R/L271M, reduced the CYP11B1 activity to about 50% when introduced individually. The removal of this unfavorable exchange leading to the single mutant L271M increased activity of CYP11B1 Pa1 approximately 3.4-fold. Two of the exchanges of the triple mutant, S168R and Q315E, did not show any or only a slightly beneficial effect on product formation, while the third exchange, M286I, alone enhanced product formation by a factor of 2.7 compared with CYP11B1 Pa1, which represents an additional slight increase compared with the parental triple mutant. The best mutant, L271M, was chosen for subsequent experiments. No significant differences in expression level in the microtiter plate were observed between CYP11B1 Pa1 and L271M, which were synthesized with 57 and 69 $nmol*L^{-1}$, respectively. This

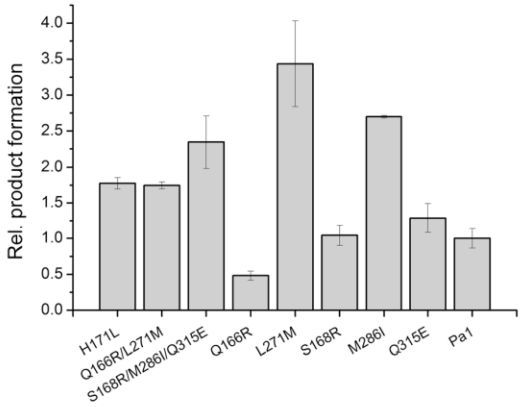

Figure 8 Relative cortisol formation of CYP11B1 enzyme variants in microtiter plates evaluated by HPLC. Mutants H171L, Q166R/L271M and S168R/M286I/Q315E were selected in the fluorescence based screening of a random mutant library. All other mutants were created by site-directed mutagenesis. Conversion took place for 48 h as described under Material and methods with 400 μM 11-deoxycortisol and cortisol formation was evaluated by HPLC and is presented in a relative manner as mean of triplicates with respective standard deviation. Product formation of CYP11B1 Pa1 was assigned as 1.

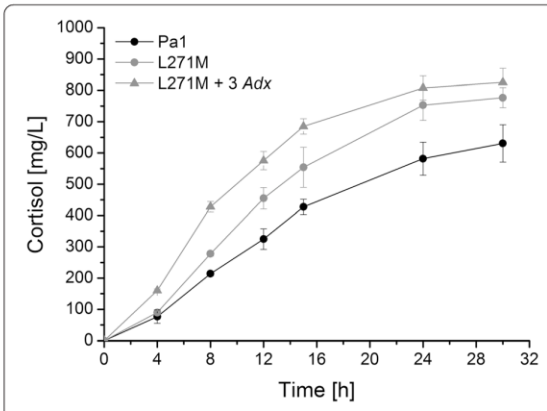

Figure 9 Time-dependent whole-cell cortisol formation by the parental CYP11B1 enzyme (Pa1) and the selected mutant L271M in shaking flasks. Pa1 (black circles) was expressed from the Twin_11B1 plasmid carrying 1 *Adx* copy, while L271M was expressed from plasmids with either 1 *Adx* (grey cirlces) or 3 *Adx* (grey triangles) copies. Reactions were performed after a 21-h expression period in TB medium with resting cells in presence of 6% DMSO and 3 mM 11-deoxycortisol. Extracted steroids were analyzed by RP-HPLC. Values represent the mean of three conversion experiments conducted in parallel with respective standard deviation.

excludes enzyme stability as underlying cause for the enhanced product yield.

Large-scale cortisol production by the selected CYP11B1 mutant

In order to verify the reliability of the microtiter plate screening for activities in larger scale, mutant L271M was analyzed for its time-dependent capacity of cortisol formation with resting cells in shaking flasks as described in Material and methods. As expected, the mutant turned out to be more productive than its parent CYP11B1 Pa1 and its application enabled an increase of the final product concentration from 631 to 777 $mg*L^{-1}$ (Figure 9). The expression level of L271M in shaking flasks was ascertained as approximately 240 $nmol*L^{-1}$ and is thus comparable to the expression level of CYP11B1 Pa1. Moreover, we combined the mutant in a plasmid with 3 *Adx* copies, as this number maximized Adx synthesis in the preceding experiments. This combination revealed an additive effect during the initial phase of the reaction and the productivity of L271M over the first 12 h was stimulated by the enhanced Adx availability in a dimension comparable to the effect of 3 *Adx* copies on CYP11B1 Pa1, leading from 38 to 48 $mg*L^{-1}*h^{-1}$ (Figure 9).

Discussion

CYP11B1, which synthesizes cortisol from 11-deoxycortisol in the human adrenal cortex, also exposes a great potential as biocatalyst in the industrial synthesis of cortisol, a pharmaceutically and thus commercially important steroid due to its anti-inflammatory and immunosuppressive effects. In this work, the successful reconstitution of a CYP11B1 system in a recombinant *E. coli* whole-cell biocatalyst, which is capable of forming cortisol from 11-deoxycortisol by 11β-hydroxylation, is presented. Because of the selectivity of 11-deoxycortisol hydroxylation by CYP11B1 in combination with a high volumetric productivity and the absence of side-product formation by *E. coli*, the developed system provides distinct advantages over other bioprocesses that have been established for cortisol production. Current industrial synthesis from 11-deoxycortisol by means of an 11β-hydroxylation via biotransformation by the fungus *C. lunata* [4] is accompanied by side-product formation [6], while alternative systems in recombinant yeast suffer from poor efficiencies [17-20]. Figure 10 compares the volumetric productivities $[mg*L^{-1}*d^{-1}]$ of publically accessible systems for cortisol production by CYP11B1 dependent biotransformation. Our new *E. coli* based system, which enabled a maximum volumetric productivity of 843 $mg*L^{-1}*d^{-1}$, exhibits a productivity which is nearly one order of magnitude higher than the best value published. In order to realize this, strategies have been developed to successfully target factors which frequently limit the application of P450s in industrial biocatalysis.

Recent studies hint at a favorable effect of N-terminal replacements of hydrophobic amino acids by positively charged ones for the expression and stability of eukaryotic P450s in *E. coli* [30,38]. The introduction of a

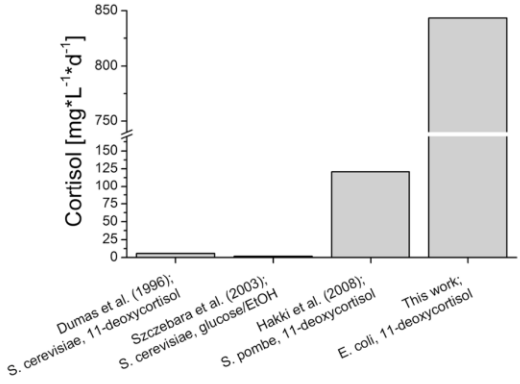

Figure 10 Comparison of the volumetric productivities of publically available CYP11B1 dependent whole-cell biocatalysts for cortisol production. The respective publications as well as the host organisms and the substrates are indicated.

hydrophilic amino acid near the N-terminus of CYP11B1 by site-directed mutagenesis of position 23 from glycine to arginine greatly enhanced the expression level, while maintaining catalytic activity, and thus improved initial productivity as well as the final product yield by a factor of 2.6.

After successfully increasing CYP11B1 expression, our second aim was an optimization of the electron flux from cellular NADPH towards CYP11B1 by engineering the redox chain which is reconstituted in *E. coli* by co-expressing AdR and Adx with the P450 from a tricistronic plasmid. Especially the electron transfer from Adx to the P450 is known to be an activity limiting step in P450 catalysis, which can be at least partially rescued by truncation and mutagenesis of Adx [31,32] or by increasing the Adx availability in the system which could already be demonstrated for CYP11B1 in in-vitro experiments with purified enzymes [21,39], as well as in recombinant cell cultures [40] and yeast [19]. In-vitro studies proof a dependency of CYP11B1 activity on Adx concentration following the Michealis-Menten equation at a stable AdR concentration [21] and describe that upon excess of Adx the maximum CYP11B1 hydroxylation activity is already achieved at molar ratios of AdR/CYP11B1 lower than 1 [41-43]. Additionally, investigations of a *class I* P450 system reconstituted in *E. coli* show a ratio for P450: ferredoxin:reductase of 1:6:1 under expression conditions optimized for substrate hydroxylation with CYP105A1 [44]. Therefore, we decided to engineer the ratio of *CYP11B1* to *Adx* expression and introduced additional copies of the Adx cDNA into the polycistronic expression unit in order to enhance *Adx* expression on transcriptional level and to reduce the rate-limiting factor of Adx availability in the *E. coli* biocatalyst. The approach succeeded to improve the productivity to a maximum possible within the polycistronic transcriptional strategy. Initial productivity was greatly accelerated and the final product concentration was increased by a factor of 1.4 at the maximum Adx level. This indicates that the limitations of the whole-cell activity caused by a reduced Adx availability were successfully overcome by the presented approach. Moreover, the high Adx concentration might have a general positive effect on the viability of the whole-cells as the [2Fe-2S]-cluster can function as a scavenger by trapping reactive oxygen species [45] which can be formed in the course of the P450 catalytic cycle [11].

In a parallel approach for the optimization of the whole-cell activity, new CYP11B1 variants with an increased activity of cortisol formation from 11-deoxycortisol were generated. Directed evolution, which consists of one or several cycles of enzyme mutagenesis, screening for the desired enzyme properties and selection of favorable mutants, represents a classical tool for such kind of enzyme engineering towards improved catalytic efficiencies, reduced uncoupling, altered selectivity or substrate specificity [46]. However, the crucial step in the establishment of a system for directed evolution is the development of a screening assay which enables a sensitive and accurate selection of the desired enzyme features with a high throughput. In order to meet these criteria, we performed a down-scale of the steroid-converting *E. coli* system to microtiter plates and employed a fluorescence based activity assay [35] in a robot-assisted manner. This assay has already been successfully applied for the improvement of the catalytic activity of CYP106A2 from *Bacillus megaterium* towards its steroidal substrates 11-deoxycortisol and progesterone [37,47]. It is premised on the fluorescence developed by steroids in an acidic, hygroscopic environment whose intensity can vary between substrate and product of a hydroxylation reaction. This is true for the transformation of 11-deoxycortisol to cortisol in the presented *E. coli* system with increasing fluorescence intensity upon formation of cortisol which proofs the applicability of the assay for the detection of activity enhanced mutants. Because of the little information about structure-activity relation of adrenocortical P450s in the literature, we conducted a random PCR mutagenesis of the entire CYP11B1 gene and examined the arising mutant library with the fluorescence screening test. We were able to select 3 mutants, H171L, Q166R/L271M and S168R/M286I/Q315E, that exhibited an approximately 2-fold increased activity in the microtiter scale and retained selectivity as shown by HPLC measurements. The activity improvements measured via fluorescence screening in the microtiter plates were supported by HPLC analysis underlining reliability of the screening procedure. The predicted localizations of mutated residues are summarized in Table 1.

The subsequent individual analysis of amino acid exchanges from the double and triple mutant revealed

Table 1 Localization of amino acid exchanges of CYP11B1 mutants with increased activity

Amino acid exchange	Localization
H171L	E'-helix, protein surface
Q166R	E'-helix, protein surface
L271M	H-helix, protein surface
S168R	E'-helix, protein surface
M286I	I-helix
Q315E	J-helix, protein surface

Localization of the respective residues discovered during the screening of a random CYP11B1 library for mutants with increased 11β-hydroxylation activity towards 11-deoxycortisol was deduced from the latest homology model of CYP11B1 [25].

activity impairing effects of mutations that introduce a positive charge into the E'-helix (S168R, Q166R), while the elimination of a potentially positively charged residue (H171L) increases the CYP11B1 activity. The removal of the unfavorable mutations further enhanced cortisol formation up to 3.4-fold compared with Pa1, when using L271M or M286I. L271M, a conservative exchange which is predicted to be localized in the H-helix on the protein surface (Figure 11), was identified as the exchange that contributes most efficiently to CYP11B1 activity. As the H-helix represents the link between the I-helix which traverses the active site and the mobile F/G-loop, which is involved in substrate access to the active site [48], the slight alteration in the residue's physicochemical properties might positively influence the flexibility of CYP11B1. M286I, the second activity increasing amino acid replacement, resides in the I-helix (Figure 11). It is not part of the active site pocket but can be assumed to strengthen structural integrity

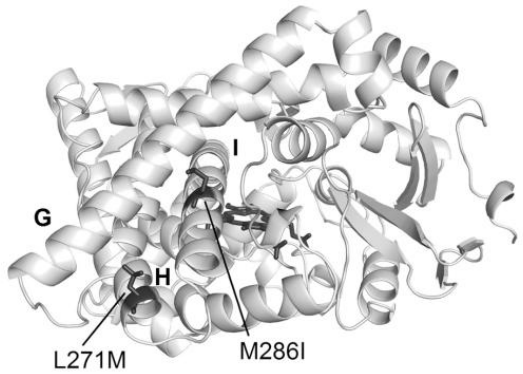

Figure 11 Localization of amino acid exchanges of CYP11B1 that increase hydroxylation activity towards 11-deoxycortisol. L271M is located on the protein surface in the H-helix, while M286I resides in the I-helix. Helices are labeled with their eponymous letter. The structure represents the latest homology model [25] and figures were created with PyMOL Builds. Mutated amino acids and the heme are highlighted in black.

of this core element. Q315E leads to the introduction of a charged group in the J-helix on the protein surface and does not have significant impact on the CYP11B1 activity which identifies M286I as determining mutation for the selection of the triple mutant. An upscale of the system from microtiter plates to shaking flasks using the most efficient mutant, L271M, could successfully reproduce the improvement of the whole-cell activity and enhanced the initial productivity as well as the final product concentration in comparison to CYP11B1 Pa1. The proposed screening procedure can thus be regarded as reliable for the optimization of large-scale processes by laboratory evolution. The mutagenesis approach can be combined with the strategy of engineering the redox partner co-expression leading to additive effects (Figure 9). However, the activity of all systems flattens after approximately 24 h and cannot be rescued with the approaches presented in this work. This points at the necessity of further optimization on the levels of process and strain engineering.

Conclusions

Taken together, we report the establishment of an *E. coli* based biocatalyst for cortisol production by a heterologous CYP11B1 system, which enables a maximum productivity of 0.84 $g*L^{-1}*d^{-1}$ under simple shaking flask conditions and thus clearly meets efficiency requirements for potential application in the pharmaceutical industry [49,50]. In total, our optimization approaches could increase the cortisol yield by a factor of 3.7. The presented strategy to overcome activity limits due to low protein ratios of Adx to CYP11B1 can be transferred to other biotechnologically interesting P450 redox chains in whole-cell application as Adx represents an efficient electron transfer partner not only for mitochondrial but also for microsomal and bacterial P450s [31,51]. The successful establishment of an accurate and fast screening system for CYP11B1 activity in combination with new structure-activity insights from the first mutant generation can be used for further directed evolution of the enzyme [52]. The system might additionally be applicable for the screening of CYP11B1 inhibitors, which can be important drugs for the treatment of for example Cushing's syndrome [53].

Material and methods

Chemicals and enzymes

All chemicals and reagents were purchased from standard sources in the highest purity available. Restriction enzymes were obtained from New England Biolabs (Ispwich, MA, USA), *Pfu* polymerase from Promega (Madison, WI, USA), and FastLink Ligase from Epicentre Biotechnologies (Chicago, IL, USA).

Bacterial strains and cultivation

Plasmid construction was performed with *E. coli* TOP10F' (*F-mcrA (mrrhsdRMS-mcrBC) f80lacZDM15 DlacX74 deoR recA1 araD139 (ara-leu)7697 galU galK rpsL (StrR) endA1 nupG*). All experiments involving protein expression and steroid conversion were conducted with *E. coli* C43(DE3) (*F– ompT gal hsdSB (rB- mB-) dcm Ion* λ). Transformation was carried out by electroporation and transformed cells were stored on agar plates supplemented with the appropriate antibiotics (100 μg/mL ampicillin and/or 50 μg/mL kanamycin) at 4°C.

Plasmid construction and mutagenesis

All methods of molecular biology were performed according to standard protocols described by [54]. The plasmid Twin_11B1 served as template for the preparation of all further plasmids. It is based on the pET-17b expression vector (MerckMillipore Novagen, Darmstadt, Germany), which enables a selection on ampicillin containing medium, and carries the cDNAs of human CYP11B1 cloned into the vector via NdeI/HindIII, bovine AdR via HindIII/KpnI and bovine Adx_{1-108} via KpnI/EcoRI in a polycistrionic transcription unit [55]. CYP11B1 was modified for expression in *E. coli* as described by [21]. Additionally, residue 29 in the *E. coli* adapted sequence (corresponding to residue 52 in the full length sequence) is mutated from leucine to methionine taking previous activity studies in recombinant fission yeast [19] into account. All utilized primers are shown in Additional file 1: Table S1.

Insertion of additional gene copies

In order to consecutively insert additional copies of the Adx cDNA into the Twin_11B1 plasmid behind the preexisting copy, the following strategy, which takes advantage of restriction site compatibility of EcoRI and MfeI, was pursued referring to [34]. In a first step, Twin_11B1 was used as a template to amplify the Adx cDNA by PCR including its ribosomal binding site and to introduce MfeI and XhoI restriction sites at the 5′ and 3′ end, respectively. The PCR product was then digested by MfeI and XhoI and ligated into the EcoRI/XhoI digested Twin_11B1 plasmid resulting in a plasmid carrying 2 Adx copies. In additional cycles of restriction and ligation further copies were inserted. In subsequent cloning attempts inserts of different Adx copy numbers could then be introduced into new plasmids by restriction and ligation via KpnI and EcoRI.

Site directed mutagenesis of CYP11B1

Targeted exchange of single amino acids was undertaken by QuikChange® mutagenesis with *Pfu* polymerase following manual instructions form Agilent Technologies (Santa Clara, USA).

Random mutagenesis of CYP11B1

Random mutagenesis of CYP11B1 was conducted by error prone PCR employing the GeneMorph II random mutagenesis kit (Stratagene, La Jolla, CA, USA). pET17b_hCYP11B1, which contains the modified cDNA of human CYP11B1 between the NdeI and HindII restriction sites of its multiple cloning site, was used as a template for the amplification of CYP11B1 with the standard primers T7 and T7term. Parameters for an average mutation frequency of 0–3 mutations per kb were chosen according to the manufacturer's protocol. The PCR product was digested by NdeI and HindIII and ligated into the likewise digested Twin_11B1 plasmid.

Whole-cell biocatalysis in shaking flasks

Protein expression

The synthesis of CYP11B1, AdR and Adx in *E. coli* took place as co-expression with the chaperone genes *GroEL* and *GroES* to ensure proper folding. Protein synthesis was carried out in 2 L Erlenmeyer flasks containing 150 mL TB medium (24 yeast extract technical, 12 g peptone, 4 mL glycerol, 4,62 g KH_2PO_4, 25 g K_2HPO_4 and distilled water ad. 1 L) supplemented with 100 μg/mL ampicillin and 50 μg/mL kanamycin. The main culture was inoculated from an overnight culture of *E. coli* C43 (DE3), that had been freshly co-transformed with the respective variant of Twin_11B1 and the chaperone vector pGro12 (kanamycin resistance and arabinose inducible promoter) [23], and was grown at 37°C and 210 rpm (Excella 25 shaker incubator, New Brunswick Scientific, Eppendorf, Ensfield, CT, USA). When an $OD_{600\ nm}$ of 0.5 was reached expression was induced by addition of 1 mM IPTG, 4 mg/mL arabinose, 1 mM of the heme precursor δ-aminolevulinic acid and 50 μg/mL ampicillin. Cultures were further incubated at 27.5°C and 200 rpm for 21 h.

Steroid conversion with resting cells

Subsequent to the expression period cultures were harvested by centrifugation (3200 *g*, 10 min, 18°C) and cells were washed in 50 mM potassium phosphate buffer (pH 7.4). Steroid conversion took place at 27.5°C and 170 rpm in 300-mL baffled flasks using 25 mL of a cell suspension of 25 g wet cell weight (wcw) per L in 50 mM potassium phosphate buffer (pH 7.4) supplemented with 1 mM IPTG, 4 mg/mL arabinose, 1 mM δ-aminolevulinic acid, 50 μg/mL ampicillin and 2% glycerol. The substrate 11-deoxycortisol (17,21-dihydroxypregn-4-ene-3,20-dione) was added from a stock solution in either EtOH, DMSO, 22.5% (m/vol) 2-hydroxypropyl-β-cyclodextrin or a 1:1 (vol:vol) mixture of EtOH and polyethyleneglycol-400. Each agent was added to the

culture in a final concentration of 6% (vol/vol). Samples were taken at defined time points.

Reversed phase HPLC analysis

For product quantification via HPLC, samples were extracted twice with one volume of chloroform. After evaporation of the organic solvent remaining steroids were suspended in acetonitrile and separated on a Jasco reversed phase HPLC system of the LC900 series (Jasco, Groß-Umstadt, Germany) using a 4.6 m × 125 mm NucleoDur C18 Isis Reversed Phase column (Macherey-Nagel, Düren, Germany) with an acetonitril/water gradient (Phase A: 10% acetonitrile, Phase B: 100% acetonitril; 0 min 20% B, 5 min 20% B, 13 min 40% B, 20 min 80% B, 21 min 80% B, 22 min 20% B, 30 min 20% B) at 40°C and a flow rate of 0.8 mL/min. Steroid pattern was monitored by an UV/Vis detector (UV-2 075 Plus, Jasco) at 240 nm.

Screening for improved CYP11B1 activity in microtiter plates

Protein expression and steroid conversion in microtiter plates as well as a fluorescence assay for the selection of CYP11B1 mutants with an improved hydroxylation activity towards 11-deoxycortisol was performed as previously reported [36], but TB medium was additionally supplemented with 50 μg/mL kanamycin and 4 mg/mL arabinose to ensure chaperone synthesis from pGro12 and did not contain a salt solution.

Analysis of protein expression

Cell lysis

For the analysis of CYP11B1 expression levels cells were harvested by centrifugation (4500 *g*, 20 min, 4°C), suspended in lysis buffer (50 mM potassium phosphate buffer (pH 7.4), 500 mM sodium-acetate, 0.1 mM EDTA, 1.5% sodium-cholate, 20% glycerol, 1.5% Tween 20, 0.1 mM phenylmethylsulfonylfluorid and 0.1 mM dithioerythritol) and disrupted with an ultrasonic homogenizer (Sonopuls HD 3200, Bandelin, Berlin, Germany). Cell debris were removed by ultracentrifugation (30000 g, 30 min, 4°C; hinac CP75, Hitachi, Tokyo, Japan) and the supernatant was surveyed for recombinant proteins.

Determination of cytochrome P450 concentration

P450 concentration was determined by CO-difference spectroscopy using a molar extinction coefficient of 91 mM^{-1} cm^{-1} as described by [56].

Western blot analysis of Adx synthesis

Cell pellets from 90 μL of the 25g_{wcw}/L cell suspension were suspended in 100 μL of SDS loading buffer (1 M Tris–HCl (pH 6.8), 40% glycerol, 20% SDS, 8% β-mercaptoethanol, 0.1% bromphenol blue) and boiled for 10 min in a water bath. Aliquots of 12 μL were separated by SDS-PAGE according to [57]. 8 μL of a 5 μM solution of purified bovine Adx_{4-108} in SDS loading buffer were applied as positive control and Protein Marker IV from PEQLAB (Erlangen, Germany) served as molecular weight standard. Proteins were blotted onto a hybond™ ECL™ nitrocellulose membrane (Amersham, GE Healthcare, UK) with the help of a semi-dry transfer system (Trans-Blot SD, Bio-Rad, Munich, Germany) and the membrane was blocked by incubation with 3% milk powder in TBS (50 mM Tris–Cl pH 7.4, 200 mM NaCl, 0.1% Tween 20) overnight. The membrane was washed 3 times for 10 minutes in fresh TBS and was incubated for 2 h with the respective polyclonal antiserum from rabbit diluted in TBS. Subsequent to 3 further washing steps in TBS the membrane was incubated for 2 h with a dilution of the horseradish peroxidase-linked goat anti rabbit IgG secondary antibody (Dako, Glostrup, Denmark) in TBS. After washing the membrane 3 times for 5 minutes with PBS (10 mM potassium phosphate buffer pH 7.4, 150 mM NaCl) staining of the antigen-antibody-complexes took place by adding 5 mg 4-chloro-1-naphtol dissolved in 2 mL ethanol and 10 μL 30% H_2O_2 in 25 mL PBS. Relative intensity of the protein bands was measured with Image Lab 3.0 from BioRad (München, Germany).

Additional file

Additional file 1: Table S1. Primers used in this work with sequence and purpose of application. Restriction sites are marked with bold letters, introduced nucleotide exchanges are underlined.

Abbreviations

AdR: Adrenodoxin reductase; Adx: Adrenodoxin; CYP11B1: 11β-hydroxylase; *E. coli*: *Escherichia coli*.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LS carried out the presented experiments and drafted the manuscript. SA established the whole-cell steriod conversion and screening conditions. AH constructed the tricistronic expression plasmids. FH participated in the design of the study, interpretation of the results and manuscript drafting. NK established the expression of CYP11B1 in *E. coli*. RB participated in the interpretation of the results and assisted in manuscript drafting. All authors read and approved the final manuscript.

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Metabolism of oral-turinabol by human steroid hormone- synthesizing cytochromes P450

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DRUG METABOLISM & DISPOSITION

Metabolism of Oral Turinabol by Human Steroid Hormone–Synthesizing Cytochrome P450 Enzymes

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ABSTRACT

The human mitochondrial cytochrome P450 enzymes CYP11A1, CYP11B1, and CYP11B2 are involved in the biosynthesis of steroid hormones. CYP11A1 catalyzes the side-chain cleavage of cholesterol, and CYP11B1 and CYP11B2 catalyze the final steps in the biosynthesis of gluco- and mineralocorticoids, respectively. This study reveals their additional capability to metabolize the xenobiotic steroid oral turinabol (OT; 4-chlor-17β-hydroxy-17α-methylandrosta-1,4-dien-3-on), which is a common doping agent. By contrast, microsomal steroid hydroxylases did not convert OT. Spectroscopic binding assays revealed dissociation constants of 17.7 μM and 5.4 μM for CYP11B1 and CYP11B2, respectively, whereas no observable binding spectra emerged for CYP11A1. Catalytic efficiencies of OT conversion were determined to be 46 min^{-1} mM^{-1} for CYP11A1, 741 min^{-1} mM^{-1} for CYP11B1, and 3338 min^{-1} mM^{-1} for CYP11B2, which is in the same order of magnitude as for the natural substrates but shows a preference of CYP11B2 for OT conversion. Products of OT metabolism by the CYP11B subfamily members were produced at a milligram scale with a recombinant *Escherichia coli*–based whole-cell system. They were identified by nuclear magnetic resonance spectroscopy to be 11β-OH-OT for both CYP11B isoforms, whereby CYP11B2 additionally formed 11β,18-diOH-OT and 11β-OH-OT-18-al, which rearranges to its tautomeric form 11β,18-expoxy-18-OH-OT. CYP11A1 produces six metabolites, which are proposed to include 2-OH-OT, 16-OH-OT, and 2,16-diOH-OT based on liquid chromatography–tandem mass spectrometry analyses. All three enzymes are shown to be inhibited by OT in their natural function. The extent of inhibition thereby depends on the affinity of the enzyme for OT and the strongest effect was demonstrated for CYP11B2. These findings suggest that steroidogenic cytochrome P450 enzymes can contribute to drug metabolism and should be considered in drug design and toxicity studies.

Introduction

In humans, most steps of steroid biosynthesis are catalyzed by monooxygenases from the cytochrome P450 (P450) superfamily, which synthesize glucocorticoids, mineralocorticoids, and sex hormones. Steroidogenesis is initiated by CYP11A1, which cleaves the side chain of cholesterol, thereby producing pregnenolone, the common precursor for all steroid hormones. In addition, CYP11A1 can convert a variety of other sterol derivatives, as well as vitamins D_2 and D_3 (Slominski et al., 2015) and a set of endogenous steroid intermediates (Mosa et al., 2015). CYP21A2 generates the substrates for gluco- and mineralocorticoid biosynthesis by its 21-hydroxylase activity. CYP11B1 and CYP11B2 subsequently synthesize gluco- and mineralocorticoids. CYP11B1 catalyzes the 11β-hydroxylation of 11-deoxycortisol yielding the glucocorticoid cortisol. CYP11B2 catalyzes hydroxylations of 11-deoxycorticosterone (DOC) in positions 11β and 18, followed by an 18-oxidation to give aldosterone, the major mineralocorticoid. CYP17A1 represents the branch point to the biosynthesis of sex hormones by its 17α-hydroxylase and 17,20-lyase activities. CYP19A1 can, finally, aromatize androgens to estrogens (Bernhardt and Waterman, 2007).

P450 catalysis requires the presence of a suitable electron transport system, which delivers the electrons necessary for the activation of molecular oxygen from the external electron donor NADPH. The mitochondrial P450 enzymes CYP11A1 and the two CYP11B isoforms depend on a class I redox system, which consists of the FAD containing NADPH-dependent ferredoxin reductase, adrenodoxin reductase (AdR), and an [2Fe-2*S*] ferredoxin, adrenodoxin (Adx). The microsomal P450 enzymes CYP17A1, CYP19A1, and CYP21A2 are supported by a single electron transfer partner (class II redox system), the NADPH-dependent cytochrome P450 oxidoreductase (CPR), which carries FMN and FAD centers (Hannemann et al., 2007).

Several studies have recently hinted at an involvement of steroidogenic P450 enzymes in the biotransformation of xenobiotic compounds, which is, according to traditional classifications of human P450 enzymes, believed to be solely conducted by microsomal isoforms from the liver (Guengerich, 2001). CYP21A2 and both CYP11B isoforms were shown to be involved in the metabolism of the synthetic anabolic androgenic steroid (AAS) metandienone (Zöllner et al., 2010; Parr et al., 2012) and there are even indications for the contribution of CYP11B1 to the

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ABBREVIATIONS: AAS, anabolic androgenic steroid; AdR, adrenodoxin reductase; Adx, adrenodoxin; CPR, cytochrome P450 reductase; DOC, 11-deoxycorticosterone; DTE, dithioerythritol; ESI, electrospray ionization; gs, gradient selected; HPLC, high-performance liquid chromatography; IMAC, immobilized metal ion affinity chromatography; LC, liquid chromatography; MR, mineralocorticoid receptor; MS, mass spectrometry; MS/MS, tandem mass spectrometry; *m/z*, mass-to-charge ratio; NMR, nuclear magnetic resonance; OT, oral turinabol (4-chlor-17β-hydroxy-17α-methylandrosta-1,4-dien-3-on); P450, cytochrome P450.

bioactivation of the nonsteroidal environmental pollutant 3-methylsulfonyl-2,2-bis(4-chlorophenyl)-1,1-dichloroethene (Lund and Lund, 1995).

In this study, we aimed to characterize the putative metabolism of the AAS oral turinabol (OT; 4-chlor-17β-hydroxy-17α-methylandrosta-1,4-dien-3-on) by steroid hormone–synthesizing P450 enzymes to further expand our understanding of their substrate specificity and possible participation in biotransformation. Anabolic agents, particularly AASs, are widely misused for doping purposes in all sports. Among them, 17α-alkylated AASs such as OT are especially popular for their oral availability, which is attributed to a reduction of the first-pass effect in the liver as a result of 17α-alkylation (Fragkaki et al., 2009). However, AASs also frequently appear in adulterated nutritional supplements, leading to an unintentional intake of AASs (Geyer et al., 2008). In antidoping controls, AASs represent the most frequently detected class of substances prohibited by the World Anti-Doping Agency (2014). Numerous side effects—including physical phenomena such as cardiovascular risks (Angell et al., 2012; Deligiannis and Kouidi, 2012) and increased risks of breast and Leydig cell cancer (Chimento et al., 2012; Sirianni et al., 2012) as well as psychiatric disorders (Palmié et al., 2013)—are attributed to AASs. Although several hypothetical models exist to describe the mechanisms behind the cardiovascular issues (Melchert and Welder, 1995; Deligiannis et al., 2006), their ability to explain all symptoms is still fragmentary. Increases in blood pressure up to hypertension are described to be secondary to increases in blood volume, which can result from a disruption of mineralocorticoid signaling (Rockhold, 1993). Mineralocorticoids, the most important of which are DOC and aldosterone in humans, regulate water and electrolyte homeostasis by controlling renal water and sodium retention as well as potassium secretion via the mineralocorticoid receptor (MR) signaling pathway (Funder, 1997). Ligand-induced activation of the cytosolic MR leads to the release of bound chaperones and nuclear localization, followed by DNA binding and the recruitment of specific coactivators, which subsequently initiates the transcription of specific target genes (Galigniana et al., 2004).

Here, we analyzed the metabolism of the xenobiotic steroid OT by human steroidogenic P450 enzymes on the molecular level to explore their drug-metabolizing capabilities. Therefore, we took advantage of the recombinant, high-yield expression of these enzymes in the bacterial host *Escherichia coli*, which only recently became feasible for all of these P450 enzymes. Dissociation constants were determined by UV-visible spectroscopy to enable a comparison with affinities toward endogenous substrates, and kinetic studies on OT metabolism were carried out using a reconstituted in vitro system with purified enzymes combined with high-performance liquid chromatography (HPLC) analysis. Metabolites were characterized by tandem mass spectrometry (MS/MS) or produced with an *E. coli* whole-cell system and purified for nuclear magnetic resonance (NMR) characterization. In addition, potential physiologic consequences of OT metabolism were investigated by demonstrating the influence of OT on the natural P450 function in a reconstituted system and by studying the effect of the metabolites on MR transactivation in a reporter gene assay.

Materials and Methods

Chemicals

All reagents were obtained from standard sources with the highest purity available. OT was kindly provided by the Center for Preventive Doping Research (German Sports University, Cologne, Germany). Other steroids were purchased from Sigma-Aldrich (St. Louis, MO). Bacterial media were purchased from Becton Dickinson (Heidelberg, Germany), and isopropyl β-D-1-thiogalactopyranoside and 5-aminolevulinic acid were from Carbolution Chemicals (Saarbrucken, Germany).

Protein Expression and Purification

Expression and Purification of P450 Enzymes. All P450 enzymes were expressed with a C-terminal polyhistidine tag from a pET-17b or pET-22b vector (Invitrogen/Life Technologies, Carlsbad, CA) in *E. coli* C43(DE) and purified as previously described with slight modifications, if necessary. The human CYP11A1 cDNA sequence (Chung et al., 1986) encoding I301 instead of M301 was modified as described by Woods et al. (1998) and was expressed and purified by immobilized metal ion affinity chromatography (IMAC) and ion exchange as presented for bovine CYP11A1 (Neunzig and Bernhardt, 2014), with slight modifications demanded for ion exchange because of differences in pI. After IMAC, the eluate was dialyzed overnight against buffer A [20 mM potassium phosphate, pH 6.8, 20% glycerol, 0.1 mM EDTA, 0.1 mM dithioerythritol (DTE), 1% sodium cholate, and 0.1% Tween 20] and applied to a SP Sepharose Fast Flow column (GE Healthcare Life Sciences, Freiburg, Germany) equilibrated with buffer A. The column was washed with buffer A, followed by buffer A containing 30 mM potassium phosphate (pH 6.8). CYP11A1 was subsequently eluted with buffer B (40 mM potassium phosphate buffer, pH 7.4, 20% glycerol, 0.1 mM EDTA, 0.1 mM DTE, 1% sodium cholate, and 0.1% Tween 20), which was replaced with buffer C (50 mM potassium phosphate buffer, pH 7.4, 20% glycerol, 0.1 mM EDTA, 0.1 mM DTE, 1% sodium cholate, and 0.05% Tween 20) by dialysis. Human CYP11B1, CYP11B2, and CYP21A2 were expressed and purified by IMAC and ion exchange as previously described by Zöllner et al. (2008), Hobler et al. (2012), and Arase et al. (2006), respectively. Human CYP17A1, modified as described by Imai (1993), was purified by IMAC according to the methods of Khatri et al. (2014a) and Petrunak et al. (2014); human CYP19A1 was purified according to the methods of Khatri et al. (2014b). Final P450 concentrations were determined by CO-difference spectroscopy (Omura and Sato, 1964) with a molar extinction coefficient (ε) of 91 mM^{-1} cm^{-1}.

Expression and Purification of Redox Partners. AdR (Sugiyama and Yamano, 1975; Sagara et al., 1993) and Adx (Uhlmann et al., 1992; Schiffler et al., 2004) were expressed in *E. coli* and purified as previously described. Concentrations were determined with ε_{450nm} of 11.3 mM^{-1} cm^{-1} for AdR and ε_{414nm} of 9.8 mM^{-1} cm^{-1} for Adx. CPR was expressed in C43(DE) as an N-terminally truncated version with a C-terminal 3-glycine-6-histidine tag and purified by IMAC as described (Sandee and Miller, 2011). The truncation of 27 amino acids at the N terminus enables high-yield expression of a soluble, catalytically active CPR. Slight modifications were made during the purification: Triton was replaced with 1% sodium cholate in all buffers and imidazole was used for washing and elution at 30 and 200 mM concentrations, respectively. The protein was finally dialyzed against buffer C described above for the removal of imidazole. The CPR concentration was determined using ε_{585nm} of 2.4 mM^{-1} cm^{-1} (Vermilion and Coon, 1978) for the semiquinone form. Bovine cytochrome b_5 was purified as reported previously by others (Neunzig et al., 2014).

In Vitro Conversion and Enzyme Activity Assay. In vitro substrate conversion was carried out at 37°C with a reconstituted system in 50 mM HEPES (pH 7.4) supplemented with 20% glycerol and 100 μM 1,2-dilauroyl-*sn*-glycero-3-phosphocholine. Prior to use, the buffer was sonicated in a sonication water bath for 5 minutes for the reconstitution of 1,2-dilauroyl-*sn*-glycero-3-phosphocholine vesicles. The system contained 0.5 μM P450, 1 mM $MgCl_2$, and 1 mM NADPH as well as a NADPH-regenerating system composed of 5 mM glucose-6-phosphate and 4 U/ml glucose-6-phosphate dehydrogenase. For mitochondrial P450 enzymes, 0.5 μM AdR and 10 μM Adx were added; 1 μM CPR was added for microsomal P450 enzymes. For CYP17A1, reactions including 2 μM bovine cytochrome b_5 were additionally performed. Substrate was added in the respective concentration from a stock solution in ethanol or in 2-hydroxypropyl-β-cyclodextrin with a final concentration of 0.225% for cholesterol. The final ethanol concentration was adjusted within each set of reactions. It was kept between 2% and 3% (2% for the kinetic studies with CYP11B1 and CYP11B2, 3% for the kinetic studies with CYP11A1 due to the higher OT concentrations required for saturation, 3% for CYP11B inhibition experiments, and 2% for CYP11A1 inhibition experiments, because cholesterol was added from a solution in cyclodextrines), whereby no effect on reaction kinetics was observed in that range. Steroids were extracted twice with chloroform, evaporated, and suspended in acetonitrile for HPLC analysis. For product quantification, progesterone was added as an internal standard prior to extraction and quantification was performed by HPLC using a calibration curve.

For OT turnover, product formation was calculated from the applied OT concentration deducting OT consumption, which was determined with a calibration curve. To monitor CYP11A1-dependent conversion of cholesterol to pregnenolone at 240 nm, the samples were boiled for 5 minutes in a water bath after the respective reaction time and a subsequent cholesterol oxidase reaction was performed for 1 hour at 37°C, which enables detection of the steroids as cholestenone and progesterone. Cortisol was used for quantification of product formation in this case.

For the determination of kinetic parameters of substrate conversion, enzyme concentrations were scaled down to 0.25 μM P450, 0.25 μM AdR, and 4 μM Adx for CYP11B1 and to 0.1 μM P450, 0.1 μM AdR, and 2 μM Adx for CYP11B2. For CYP11A1, the conditions described above were maintained. Reactions were stopped under steady-state conditions by freezing in liquid nitrogen. Reaction times were between 2 and 15 minutes for CYP11B1 and CYP11B2 and between 20 and 25 minutes for CYP11A1.

HPLC/UV-Visible Detection

Steroids were separated on a Jasco reversed-phase HPLC system (Jasco, Gross-Umstadt, Germany) using a 4.6 × 125 mm NucleoDur C18 Isis reversed-phase column (Macherey-Nagel, Dueren, Germany) with an acetonitrile/water gradient at 40°C and a flow rate of 0.8 ml/min. The steroid pattern was monitored by an UV-visible detector (UV-2 075 Plus; Jasco) at 240 nm.

Spectroscopic Binding Assay

The determination of dissociation constants was performed by difference spectroscopy using tandem cuvettes as described (Schenkman, 1970) with a Jasco V-630 spectrophotometer. CYP11B1 or CYP11B2 (1 μM) diluted in 50 mM potassium phosphate buffer (pH 7.4) supplemented with 20% glycerol, 0.5% sodium cholate, and 0.05% Tween20 was titrated with increasing concentrations of the steroid from stock solutions in dimethylsulfoxide and difference spectra were recorded from 350 to 500 nm. Titrations were performed three times. To determine the binding dissociation constant (K_d), the averaged ΔA (peak-to-trough absorbance difference) was plotted against the ligand concentration. Plots were fitted with Origin 8.6 software (OriginLab Corporation, Northampton, MA) by either hyperbolic regression [$\Delta A = (A_{max}[S]/K_d + [S])$] or a tight binding quadratic equation [$\Delta A = (A_{max}/2[E])\{(K_d + [E] + [S]) - \{(K_d + [E] + [S])^2 - 4[E][S]\}^{1/2}\}$], whereby ΔA represents the peak-to-trough absorbance difference at every ligand concentration, A_{max} is the maximum absorbance difference at saturation, [E] is the enzyme concentration (1 μM), and [S] is the substrate concentration. To measure binding spectra of CYP11A1, a 5-μM concentration of the enzyme was used and 5 μM Adx was added to enhance the spectroscopic signal.

E. coli–Based Whole-Cell Product Formation and Product Purification

Large-scale substrate conversion by human CYP11B2 was conducted with a recombinant *E. coli* whole-cell system as previously described for CYP11B1 (Schiffer et al., 2015b). Briefly, *E. coli* C43(DE3) [*F– ompT gal hsdSB (rB− mB−) dcm ion* λ] cells were transformed by electroporation with the pET-17b–based plasmid Twin_11B2 encoding human CYP11B2, bovine AdR, and bovine $Adx_{1–108}$ and the plasmid pGro12 for the cosynthesis of the molecular chaperones GroEL/ES (Nishihara et al., 1998). Terrific broth media (150 ml; 24 g yeast extract technical, 12 g peptone, 4 ml glycerol, 4.62 g KH_2PO_4, 25 g K_2HPO_4, and 1 liter of distilled water) were supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin in a 2-liter Erlenmeyer flask and were inoculated from an overnight culture. Cultures were incubated at 37°C and 210 rpm until an optical density at 600 nm of 0.5 was reached. Protein expression was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside, 1 mM δ-aminolevulinic acid, 4 mg/ml arabinose, and 50 μg/ml ampicillin and took place at 27.5°C and 200 rpm for 24 hours. For subsequent substrate conversion, cells were harvested by centrifugation (3200 × *g*, 10 minutes, 18°C), washed in 50 mM potassium phosphate buffer (pH 7.4), and suspended in 150 ml buffer supplemented with 1 mM isopropyl β-D-1-thiogalactopyranoside, 4 mg/ml arabinose, 1 mM δ-aminolevulinic acid, 50 μg/ml ampicillin, and 2% glycerol in a 2-liter Erlenmeyer flask. OT was added from a stock solution in ethanol to a final concentration of 100 μM and conversion was performed at 27.5°C and 200 rpm for 24 hours. Steroids were extracted twice with one culture volume of ethyl acetate and the organic phase was evaporated to dryness. Steroids were suspended in acetonitrile and separated on a Jasco reversed-phase HPLC system with a NucleoDur 100-5 5 × 250 mm C18 EC column (Macherey-Nagel) and an acetonitrile/water gradient at a flow rate of 3 ml/min and 40°C. Steroids were monitored at 240 nm and fractions containing the desired products were collected, evaporated to dryness, and analyzed by NMR and liquid chromatography (LC)/mass spectrometry (MS).

LC-MS/MS Quadrupole Time-of-Flight Setup

High-resolution/high-accuracy LC-MS/MS measurements were conducted using an Agilent 6550 iFunnel quadrupole time-of-flight LC-MS/MS instrument (Waldbronn, Germany) equipped with a dual Agilent Jet Stream electrospray ionization (ESI) source operated at a gas temperature of 290°C and an ionization voltage of 3500 V in positive mode. The mass spectrometer was calibrated using the manufacturer's protocol allowing for mass errors < 5 ppm for the period of analysis. The mass analyzer acquired data from mass-to-charge (*m/z*) ratios of 50 to 600 with an acquisition time of 200 milliseconds per spectrum, and collision energies of MS/MS experiments were adjusted between 15 and 25 eV. LC was accomplished by means of an Agilent 1290 Infinity LC system equipped with an Agilent Eclipse XDB-C18 column (5 μm, 4.6 × 150 mm) protected by a guard column of the same material. The eluents were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) run at a flow rate of 1 ml/min, enabling gradient elution of the analytes starting at 98% A, decreasing to 0% A within 14 minutes, followed by a 4-minute re-equilibration period at starting conditions.

NMR Characterization of the Major Metabolites

The NMR spectra were recorded in $CDCl_3$ with a Bruker DRX 500 or a Bruker Avance 500 NMR spectrometer at 298 K (Bruker, Billerica, MA). The chemical shifts were relative to $CHCl_3$ at δ 7.26 (^{1}H NMR) and $CDCl_3$ at δ 77.00 (^{13}C NMR), respectively, using the standard δ notation in parts per million. The one-dimensional NMR spectra (^{1}H and ^{13}C NMR, Distortionless Enhancement by Polarization Transfer measurements with 135°) and the two-dimensional NMR spectra (gradient selected (gs)-HH-Correlated Spectroscopy, gs-Nuclear Overhauser Enhancement Spectroscopy, gs-Heteronuclear Single Quantum Correlation, and gs-Heteronuclear Multiple Bond Correlation) were recorded using the Bruker pulse program library. All assignments were based on extensive NMR spectral evidence.

MR Transactivation Assay

Steroids were analyzed for their ability to activate the human MR applying the Human Mineralocorticoid Receptor Reported Assay System (INDIGO Biosciences, State College, PA) following the manufacturer's protocol in a dose-dependent manner with concentrations from 2 to 20,000 pM. Aldosterone, which was used as positive control, was supplied with the assay.

Results

In Vitro Metabolism Assay of OT by Human Steroidogenic P450 Enzymes

Conversion of OT by the human steroid hormone–synthesizing P450 enzymes was assayed with recombinant proteins purified from *E. coli* as previously described (for references, see the *Materials and Methods*). The natural electron transfer chain from NADPH to P450 was reconstituted with human Adx and AdR for mitochondrial P450 enzymes and CPR for the microsomal P450 enzymes, and the reaction was supported by an NADPH-regenerating system. Putative product formation was analyzed by HPLC and is summarized in Table 1. It demonstrated OT metabolism by CYP11A1 as well as by the two isoforms of the CYP11B subfamily. By contrast, the microsomal P450 enzymes CYP17A1, CYP19A1, and CYP21A2, did not show any conversion of this AAS. CYP11B1- and CYP11B2-dependent conversion of OT showed distinct but partially overlapping product patterns (Fig. 1). Metabolites with the same retention times in the HPLC

TABLE 1

Metabolism of OT by human steroidogenic P450 enzymes

OT conversion was assayed in a reconstituted in vitro system with NADPH and the natural redox system (mitochondrial: AdR and Adx; microsomal: CPR) and was analyzed by HPLC.

P450 Enzyme	Redox System	OT Conversion	Control Reaction
CYP11A1	Mitochondrial	+	Cholesterol → pregnenolone
CYP11B1	Mitochondrial	+	DOC → corticosterone
CYP11B2	Mitochondrial	+	DOC → corticosterone
CYP17A1	Microsomal	−	Progesterone → 17-hydroxyprogesterone
	Microsomal plus cytochrome b_5	−	17-hydroxyprogesterone → androstenedione
CYP19	Microsomal	−	Androstenedione → estrone
CYP21A2	Microsomal	−	Progesterone → DOC

Product formation is indicated by a plus sign, and no product forming activity is indicated by a minus sign. A positive control was performed with a natural substrate.

measurements are assumed to be the same OT derivatives. Whereas CYP11B1 forms one main metabolite (1) as well as two side products (2 and 3) in minor amounts, CYP11B2 produces three main products (1, 4, and 5) and three intermediate (2) or side products (3 and 6). For the CYP11B2-catalyzed reaction, time dependence of the product pattern could be observed. Peak area portions of metabolites 1 and 2 are reduced over the time, whereas those of metabolites 4 and 5 increase (Fig. 1, C and D), which suggests that metabolites 4 and 5 are formed from 1 and 2 in a follow-up reaction. CYP11A1 converted OT rather unselectively to one main product (metabolite 9) and several side products (metabolites 7, 8, and 10–12; HPLC chromatogram not shown). Because product formation was weak compared with CYP11B1 and CYP11B2 and a preparative setup did not seem to be feasible, we subsequently used an LC-MS/MS approach to obtain the maximal information about the metabolites of the CYP11A1-catalyzed conversion of OT.

LC-MS/MS Analysis of CYP11A1-Dependent OT Metabolism

The mixture of in vitro CYP11A1-derived metabolites was analyzed by LC-MS/MS using full-scan and product ion scan experiments. By means of the accurate masses of mono- and dihydroxylated analogs to OT as well as the consideration of diagnostic product ions generated from protonated molecules of the observed analytes, several different metabolic products were identified. The extracted ion chromatograms of mono- and dihydroxylated OT are illustrated in Fig. 2, suggesting the formation of at least six metabolites (metabolites 7–12). The product ion mass spectrum of metabolite 7 is depicted in Fig. 3A, presenting several product ions indicative for an unmodified steroidal A/B-ring system such as *m/z* 155, 169, and 181 in accordance to literature data (Thevis and Schänzer, 2005; Pozo et al., 2008), which support assigning metabolite 7 to C- or D-ring hydroxylated OT. Metabolite 8 also yielded product ions at *m/z* 155, 169, and 181 similar to metabolite 7 but at substantially different abundances (Fig. 3B). Moreover, intense product ions at *m/z* 205

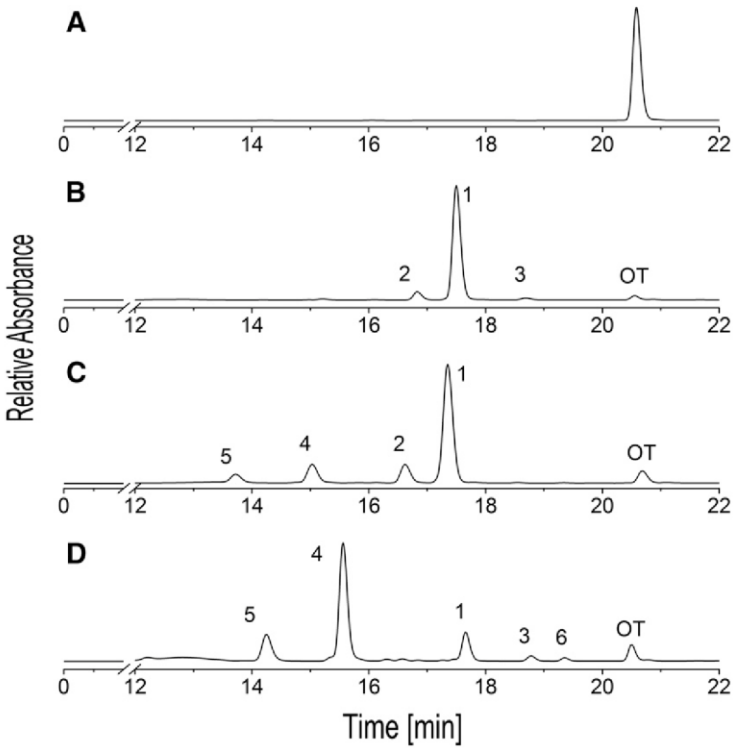

Fig. 1. Reversed-phase HPLC chromatograms of the in vitro conversion assays of OT by human CYP11B1 and CYP11B2. OT (100 μM) was incubated at 37°C with a reconstituted P450 system consisting of 0.5 μM P450, 10 μM Adx, 0.5 μM AdR, 1 mM NADPH, and an NADPH-regenerating system. (A) Incubation without NADPH for 30 minutes as the negative control. (B) CYP11B1-dependent conversion of OT for 30 minutes. (C and D) CYP11B2-dependent conversion of OT for 10 minutes and 30 minutes, respectively.

and 207 complemented this spectrum, which were suggested to also originate from the steroidal A/B-ring based on the observed accurate masses and corresponding elemental compositions ($C_{12}H_{10}ClO$ and $C_{12}H_{12}ClO$, respectively). However, in the absence of further information, a structural assignment of this analyte was not possible. The product ion mass spectrum of metabolite 9 contained a characteristic ion at *m/z* 171 (Fig. 3C), representing the counterpart to *m/z* 155 after hydroxylation. Hence, the location of the hydroxyl function is postulated to be within the A/B-ring moiety of the steroid. Noteworthy, both metabolites 7 and 8 showed two subsequent water eliminations to yield a product ion at *m/z* 315.149, whereas metabolite 9 predominantly generated a product ion at *m/z* 315.195 as a result of a HCl elimination (Fig. 3C, inset), which further corroborated an A/B-ring modification of this analyte.

In addition to the aforementioned monohydroxylated metabolites of OT, dihydroxylated species were also observed by means of the accurate mass of the protonated molecules (Fig. 2, compounds 10–12). Because of the comparably low abundance of these analytes and the limited amount of features identifiable by means of ESI-MS/MS experiments, further studies into the structural compositions were conducted only for metabolite 11 (Fig. 3D). Here, the same product ion as observed for product 9 was observed at *m/z* 171, suggesting the location of one hydroxyl function at the A/B-ring of the metabolite. In addition, the protonated molecule at *m/z* 367 was found to release HCl (36 Da), followed by two consecutive water losses to yield *m/z* 331, 313, and 295, respectively. Assuming that the significant proton affinity of the conjugated π-electron system of the A-ring leads to charge-remote elimination processes, a location of the second hydroxyl function at the steroidal C/D-ring system is likely.

Determination of Dissociation Constants and Kinetic Parameters

To assess the efficiency of OT metabolism by the human CYP11 family members, in vitro characterization of substrate affinity and reaction kinetics was performed. For CYP11B1 and CYP11B2, a high-spin shift of the heme iron was observed upon OT binding. This feature was used to determine dissociation constants (K_d) from the type I difference spectra recorded during the titration of P450 with increasing concentrations of OT (Fig. 4). CYP11B2 showed a K_d value of 5.4 $\pm$ 0.4 μM, whereas CYP11B1 has less affinity for OT with a K_d of 17.7 $\pm$ 2.2 μM. CYP11A1, however, showed a putative type II–like difference spectrum with a minimum around 405 nm and a maximum between 422 and 424 nm, which was not quantifiable even under high P450 concentrations and in the presence of Adx (Fig. 4C). The observation of a type II difference spectrum with OT is unexpected because as all other type II ligands described in the literature, to our knowledge, possess a nitrogen atom, whose association with the heme iron induces a low spin shift. However, the spin-state equilibrium can also be influenced by distortions of the porphyrin molecule (Groenhof, 2007). Because of the small size of OT compared with cholesterol, two molecules of OT might bind in the active site, resulting in a very close position of one OT molecule above the heme. Putative emerging substrate-induced heme deformation could then lead to a spin-state crossover toward the low-spin state.

Moreover, we determined kinetic parameters of OT conversion by performing in vitro reactions under steady-state conditions and quantification of OT consumption (Fig. 5). Molar ratios of P450 and its redox partners were 1:20:1 P450/Adx/AdR, because the excess of Adx excludes the electron transfer to P450 as a limiting step and maximum activities of

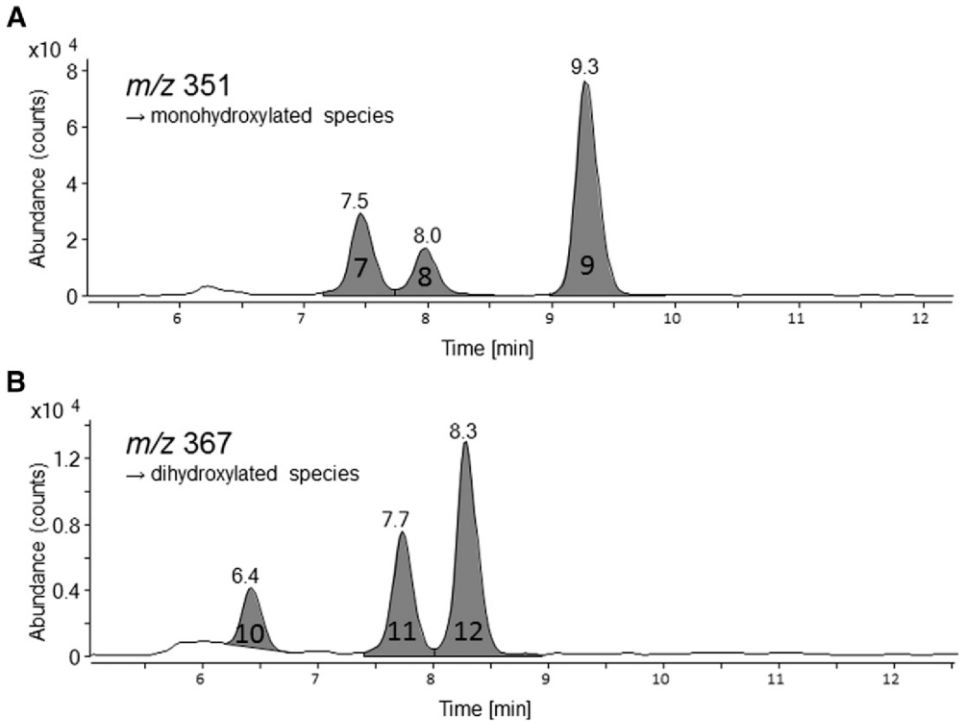

Fig. 2. Extracted ion chromatograms of CYP11A1-derived mono- and dihydroxylated OT. OT (100 μM) was incubated at 37°C with a reconstituted P450 system consisting of 0.5 μM CY11A1, 10 μM Adx, 0.5 μM AdR, 1 mM NADPH, and an NADPH-regenerating system. (A and B) Protonated molecules of steroids $[M+H]^+$ as measured by LC-ESI-MS are shown with metabolites 7, 8, and 9 representing monohydroxylated OT (*m/z* 351) (A) and metabolites 10, 11, and 12 representing dihydroxylated OT (*m/z* 367) (B).

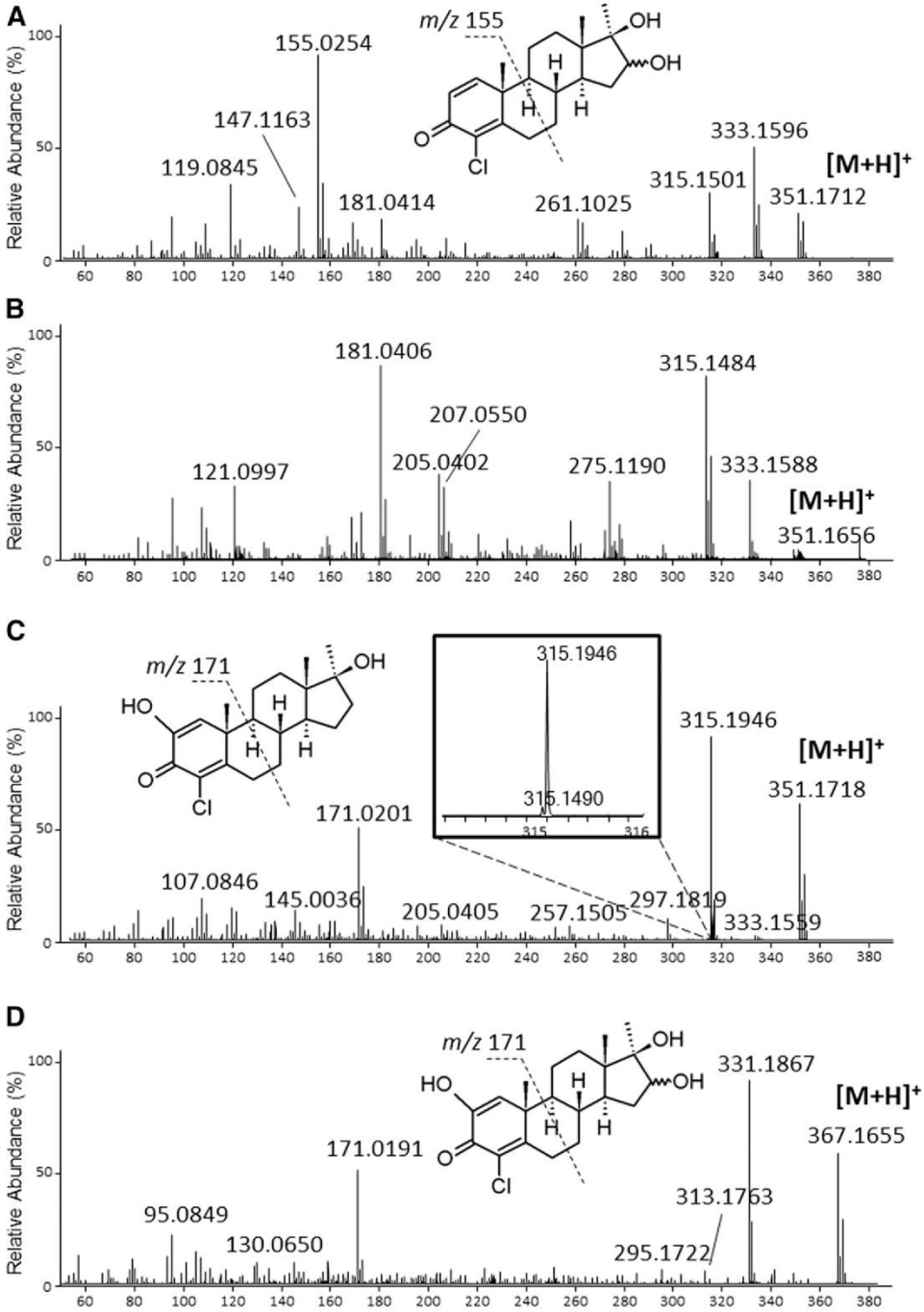

Fig. 3. Product ion mass spectra of protonated CYP11A1-derived OT products at *m/z* 351 and *m/z* 367. (A–D) Product ion mass spectra of product 7 (*m/z* 351, collision energy: 15 eV; RT = 7.5 minutes) (A), product 8 (*m/z* 351, collision energy: 15 eV; RT = 8.0 minutes) (B), product 9 (*m/z* 351, collision energy: 25 eV; RT = 9.3 minutes) (C), and product 11 (*m/z* 367, collision energy: 25 eV; RT = 7.7 minutes) (D). Proposed structures of the respective OT metabolites are shown. Fragmentation and the resulting fragment ion masses are indicated. RT, retention time.

CYP11 systems are observed at P450/AdR ratios ≥ 1 upon Adx excess (Seybert et al., 1978, 1979; Lambeth et al., 1982). For CYP11B2, formation of the downstream products was approximately 20% under these reaction conditions, so that the parameters primarily describe the first hydroxylation reaction. The resulting parameters K_M, k_{cat}, and the catalytic efficiency k_{cat}/K_M are summarized in Table 2.

Influence of OT on Natural Substrate Conversion

The binding and metabolism of OT at relevant catalytic rates by the three enzymes suggests possible interference with the conversion of their natural substrates. In vitro conversions using cholesterol as substrate for CYP11A1 and DOC for CYP11B1 and CYP11B2 in the presence of increasing OT concentrations demonstrate the capability of

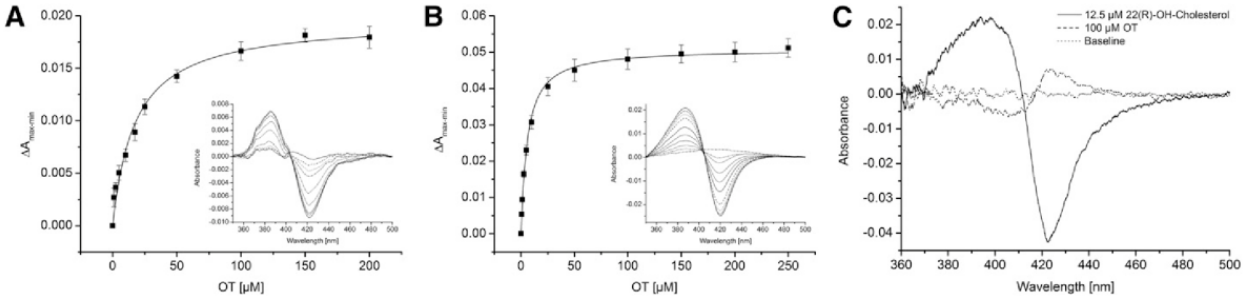

Fig. 4. Spectroscopic characterization of OT binding by the CYP11 family. (A and B) Determination of OT affinity to CYP11B1 (A) and CYP11B2 (B) by difference spectroscopy and regression of the absorbance shift. The respective CYP11B isoform (1 μM) was titrated with increasing concentrations of OT in tandem cuvettes and difference spectra were recorded from 350 to 500 nm (insets). Peak to trough differences of the resulting type I spectra were plotted against the OT concentration and dissociation constants were determined by hyperbolic regression for CYP11B1 (R^2 = 0.98) and a tight binding quadratic equation for CYP11B2 (R^2 = 0.99). Measurements were performed in triplicate. (C) Difference spectra of CYP11A1 upon addition of OT and 22(*R*)-OH-cholesterol. CYP11A1 (5 μM) was supplemented with its substrates in saturating concentrations in the presence of Adx at equimolar concentration.

OT to inhibit steroid biosynthesis on an enzymatic level (Fig. 6). Product formation was inhibited for all three enzymes in a concentration-dependent manner, with a stronger effect on CYP11B2 than on CYP11B1 and CYP11A1, which are influenced to a comparable extent. With a 5-fold excess of OT over the respective natural substrate, CYP11A1- and CYP11B1-dependent product formation was reduced to 42% and 49%, respectively, whereas CYP11B2-dependent product formation was reduced as low as 12%. The regulation of steroid biosynthesis is also the result of signaling on multiple levels (hypothalamic-pituitary-adrenal axis, renin-angiotensin system, etc.). A deeper characterization of the inhibitory effects of OT was thus not considered as conducive for the interpretation of their relevance.

E. coli–Based Whole-Cell OT Conversion

Preparative scale production of the OT metabolites formed by the CYP11B isoforms for NMR characterization and further investigation of the major metabolites was performed with an *E. coli*–based whole-cell conversion system as previously reported for CYP11B1-dependent transformation of 11-deoxycortisol to cortisol (Schiffer et al., 2015b). Because CYP11B1 products are also formed by CYP11B2, application of CYP11B2 was chosen. The complete redox chain was transferred into *E. coli* by coexpression of CYP11B2, bovine AdR, and truncated bovine $Adx_{1–108}$ from a tricistronic plasmid. Functional folding was supported by the overexpression of the molecular chaperones GroEL/ES (Nishihara et al., 1998). The CYP11B2 whole-cell biocatalyst was applied for the conversion of 100 μM OT under nongrowing conditions to maximize the availability of NADPH for the CYP11B2 reaction. After 24 hours, approximately 90% of OT was converted with the same product pattern as observed in vitro (data not shown). Individual yields for the three major products as estimated by peak area portions were about 30%, 33%, and 23% for metabolites 1, 4, and 5, respectively. All three products were purified in milligram amounts by preparative HPLC.

NMR Characterization of the OT Metabolites

Purified products were structurally characterized by NMR spectroscopy. Elucidated structures are illustrated in Fig. 7.

Metabolite 1. Compared with OT, the ^{1}H and ^{13}C NMR spectra of its conversion metabolite 1 [11β-OH-OT (4-chlor-11β,17β-dihydroxy-17α-methylandrosta-1,4-dien-3-one)] showed signals for an additional secondary hydroxyl group (δ_H 4.44 td; δ_c 70.36), which could be located at C-11 by means of two-dimensional NMR. For example, its proton showed correlations to H-9 (δ 1.07, dd), H-12a (δ 1.57, dd), and H-12b (δ 1.64, dd) in HH-Correlated Spectroscopy. H-11 must be in an equatorial position because of its small coupling constants to H-9 (J = 3.7 Hz) and to H-12 (J = 2.5 and 3.7 Hz) and is therefore in α orientation. Consequently, the hydroxyl at C-11 is β orientated. ^{1}H NMR ($CDCl_3$, 500 MHz) data were as follows: δ 1.07 (dd, 11.0 and 3.7 Hz, H-9), 1.08 (m, H-7a), 1.13 (m, H-14), 1.15 (s, 3xH-20), 1.18 (s, 3xH-18), 1.44 (qd, 12.0 and 6.5 Hz, H-15a), 1.53 (s, 3xH-19), 1.57 (dd, 14.2 and 3.7 Hz, H-12a), 1.64 (dd, 14.2 and 2.5 Hz, H-12b), 1.65 (m, H-15b), 1.76 (ddd, 14.0, 9.5, and 6.5 Hz, H-16a), 1.85 (ddd, 14.0, 12.0, and 3.5 Hz, H-16b), 2.11 (m, H-7b), 2.14 (m, H-8), 2.39 (td, 13.8 and 5.3 Hz, H-6a), 3.24 (ddd, 13.8, 4.8, and 2.3 Hz, H-6b), 4.44 (td, 3.7 and 2.5 Hz, H-11), 6.39 (d, 10.0 Hz, H-2),

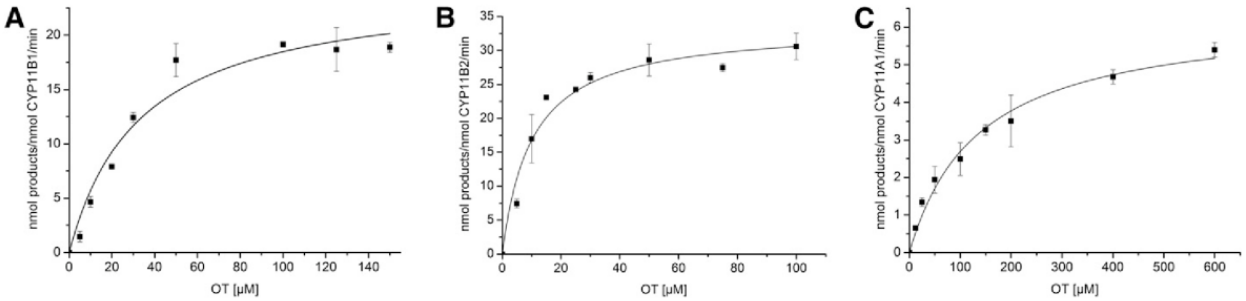

Fig. 5. Determination of kinetic parameters for OT conversion by human CYP11B1 (A), CYP11B2 (B), and CYP11A1 (C) in a reconstituted in vitro system. The system was reconstituted with the respective P450, Adx, and AdR at a ratio of 1:20:1. Reactions took place at 37°C in presence of an NADPH-regenerating system and were stopped under steady-state conditions. OT consumption was determined by HPLC using progesterone or cortisol as the internal standard. All values represent the mean of triplicates with the standard deviation. Kinetic parameters were ascertained by hyperbolic regression (R^2 = 0.96 for CYP11B1 and R^2 = 0.98 for CYP11A1 and CYP11B2).

TABLE 2

Kinetic parameters of OT conversion by CYP11B1, CYP11B2, and CYP11A1 determined as shown in Fig. 5

P450 Enzyme	K_M	k_{cat}	k_{cat}/K_M
	μM	min^{-1}	$min^{-1}\ mM^{-1}$
CYP11B1	33 ± 9	25 ± 2	741
CYP11B2	10 ± 2	34 ± 2	3338
CYP11A1	136 ± 21	6 ± 0.4	46

and 7.32 (d, 10.0 Hz, H-1). ^{13}C NMR ($CDCl_3$, 125 MHz) data were as follows: δ 16.42 (CH_3, C-18), 21.42 (CH_3, C-19), 23.43 (CH_2, C-15), 25.87 (CH_3, C-20), 28.23 (CH_2, C-6), 32.05 (CH, C-8), 32.40 (CH_2, C-7), 38.67 (CH_2, C-16), 41.00 (CH_2, C-12), 44.78 (C, C-13), 46.79 (C, C-10), 50.94 (CH, C-14), 56.22 (CH, C-9), 70.36 (CH, C-11), 81.76 (C, C-17), 126.62 (CH, C-2), 127.23 (CH, C-4), 155.84 (CH, C-1), 163.37 (C, C-5), and 178.57 (C, C-3).

Metabolite 4. The NMR spectra of metabolite 4 [11β, 18-di-OH-OT (4-chlor-11β,17β,18-trihydroxy-17α-methylandrosta-1,4-dien-3-one)] were similar to those of metabolite 1, especially for the resonances of C-11 (δ_C 69.12) and H-11 (δ_H 4.39 q, 3.3 Hz) as well as the coupling pattern of H-11; however, P2 lacked resonances for methyl C-18. Therefore, signals for a primary hydroxyl group at δ_H 4.00 (d, 11.3 Hz), 4.04 (d, 11.3 Hz), and δ_C 63.49 appeared in the spectra. Data thus obtained led to the 11β, 18-dihydroxy derivative of OT. Two-dimensional NMR measurements supported the structure and led to the full assignments. ^{1}H NMR ($CDCl_3$, 500 MHz) data were as follows: δ 1.03 (dd, 11.3 and 3.3 Hz, H-9), 1.05 (m, H-7a), 1.20 (s, 3xH-20), 1.21 (m, H-14), 1.29 (m, H-12a), 1.44 (m, H-15a), 1.54 (s, 3xH-19), 1.69 (m, H-15b), 1.82 (ddd, 14.0, 9.5, and 7.0 Hz, H-16a), 2.08 (m, H-16b), 2.09 (m, H-7b), 2.20 (m, H-8), 2.33 (m, H-12b), 2.39 (td, 14.0 and 5.4 Hz, H-6a), 3.25 (ddd, 14.0, 4.8, and 2.7 Hz, H-6b), 4.00 (d, 11.3 Hz, H-18a), 4.04 (d, 11.3 Hz, H-18b), 4.39 (q, 3.3 Hz, H-11), 6.39 (d, 10.0 Hz, H-2), and 7.38 (d, 10.0 Hz, H-1). ^{13}C NMR ($CDCl_3$, 125 MHz) data were as follows: δ 21.16 (CH_3, C-19), 23.97 (CH_2, C-15), 27.04 (CH_3, C-20), 28.21 (CH_2, C-6), 32.07 (CH, C-8), 32.70 (CH_2, C-7), 34.19 (CH_2, C-12), 39.21 (CH_2, C-16), 46.90 (C, C-10), 48.31 (C, C-13), 50.38 (CH, C-14), 56.50 (CH, C-9), 63.49 (CH_2, C-18), 69.12 (CH, C-11), 83.49 (C, C-17), 126.44 (CH, C-2), 127.24 (CH, C-4), 156.10 (CH, C-1), 163.08 (C, C-5), and 178.57 (C, C-3).

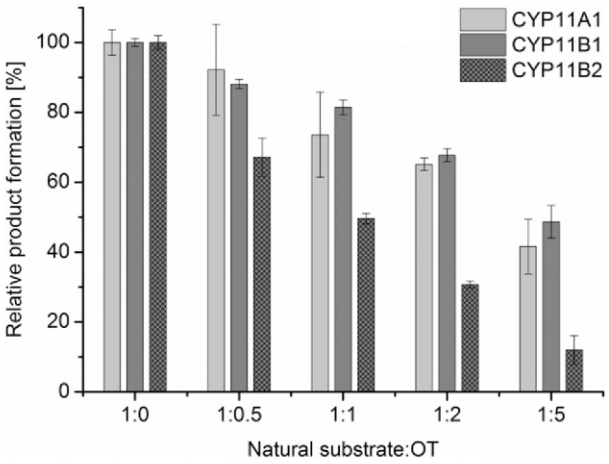

Fig. 6. Relative product formation from the natural substrates in the presence of OT in a reconstituted in vitro system. The system consisted of 0.5 μM P450, 10 μM Adx, 0.5 μM AdR, 1 mM NADPH, and an NADPH-regenerating system and the reaction took place at 37°C for 1 minute for the CYP11B isoforms and 10 minutes for CYP11A1. DOC was used as a substrate for CYP11B1 and CYP11B2, and cholesterol was used for CYP11A1. The substrate concentration was 20 μM to ensure conditions below the saturation but in excess over P450 and OT was added in the indicated ratios. DOC consumption or pregnenolone formation was determined by HPLC using progesterone or cortisol, respectively, as an internal standard. Each bar represents the mean of three reactions with the respective standard deviation.

Metabolite 5. The NMR spectra of metabolite 5 [11β, 18-epoxy-18-OH-OT (4-chlor-11β, 18-epoxy-17β,18-dihydroxy-17α-methylandrosta-1,4-dien-3-one)] revealed a (11-18)-hemi-acetal function. This was obvious by the characteristic acetal resonance for C-18 (δ_C 99.40, CH) and its correlation with the hydroxyl proton H-11 (δ_H 4.77 d) in the HMBC. Thus, the structure of metabolite 5 represented the tautomeric form of the 11β-hydroxy-18-aldehyde derivate of OT. ^{1}H NMR ($CDCl_3$, 500 MHz) data were as follows: δ 1.04 (d, 10.7 Hz, H-9), 1.10 (m, H-7a), 1.28 (s, 3xH-20), 1.49 (m, H-14), 1.28 (d, 11.5 Hz, H-12a), 1.53 (m, H-15a), 1.36 (s, 3xH-19), 1.85 (m, H-15b), 1.95 (m, H-16a and H-16b), 2.07 (m, H-7b), 1.85 (m, H-8), 2.30 (td, 13.7 and 4.8 Hz, H-6a), 2.42 (dd, 11.5 and 6.5 Hz, H-12b), 3.34 (dt, 13.7 and 3.5 Hz, H-6b), 5.32 (s, H-18), 4.77 (d, 6.5 Hz, H-11), 6.40 (d, 10.0 Hz, H-2), and 7.14 (d, 10.0 Hz, H-1). ^{13}C NMR ($CDCl_3$, 125 MHz) data were as follows: δ 20.40 (CH_3, C-19), 23.34 (CH_2, C-15), 27.40 (CH_3, C-20), 28.83 (CH_2, C-6), 31.07 (CH_2, C-7), 35.27 (CH_2, C-12), 36.92 (CH, C-8), 39.51 (CH_2, C-16), 45.64 (C, C-10), 48.01 (CH, C-14), 55.32 (CH, C-9), 57.19 (C, C-13), 75.65 (CH, C-11), 78.64 (C, C-17), 99.40 (CH, C-18), 126.63 (CH, C-2), 128.94 (CH, C-4), 154.46 (CH, C-1), 161.42 (C, C-5), and 178.13 (C, C-3).

MR Activation Assay with the CYP11B-Derived OT Metabolites

CYP11B-derived OT metabolites carry the same oxy-functionalizations as are introduced into the steran scaffold during the biosynthesis of natural mineralocorticoids. Therefore, we evaluated their potential to activate the human MR to investigate putative new or altered functions of the OT metabolites. The assay was performed with a commercially available cell culture–based system, which gives a luminescence signal upon activation of the MR. Purity of the test compounds was verified by LC-MS prior to the assay. Test concentrations ranged from 2 to 20,000 pM, which resulted in full dose response for the natural MR ligand aldosterone. The respective EC_{50} value of 42 pM is consistent with the range of the value given in the manufacturer's protocol. A detectable MR activation by OT and its metabolites was observed only with the highest assay concentration and represented only 30% of the maximum aldosterone response (Fig. 8). The modifications at C_{11} and C_{18} introduced by CYP11B2 did not alter the agonist potential of OT.

Discussion

Human P450 enzymes are traditionally classified into a group of drug-metabolizing P450 enzymes expressed in the liver and those that carry out the biosynthesis of endogenous compounds such as steroid hormones. In this study, we examined whether the second group might additionally contribute to the metabolism of xenobiotics by investigating the synthetic steroidal drug OT, which is a common doping agent. All six steroidogenic P450 enzymes were tested for their activity toward OT. Although no conversion of OT was found using CYP17A1, CYP19A1, and CYP21A2, the three mitochondrial P450 enzymes (CYP11A1 and both isoforms of the CYP11B subfamily) efficiently catalyze conversion of OT with an affinity and catalytic efficiency in the same order of magnitude as for their natural substrates. CYP11B2 binds OT with a K_d only approximately 4-fold higher than that for the natural substrate DOC (reported to be 1.34 μM; Hobler et al., 2012), and the catalytic efficiency (k_{cat}/K_M) of OT conversion by CYP11B1 and CYP11B2 is even slightly higher than previously determined for the natural substrates (Zöllner et al., 2008; Hobler et al., 2012). It is striking

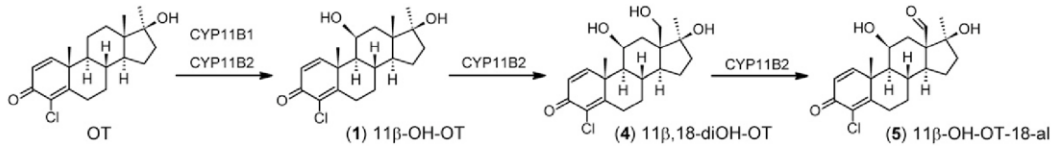

Fig. 7. Hydroxylation and oxidation of OT catalyzed by the human CYP11B subfamily. Structures were revealed by NMR spectroscopy after P450-dependent whole-cell catalysis in *E. coli.*

that CYP11B2 shows a higher activity and affinity for OT than CYP11B1. It was postulated that the extended functional spectrum of CYP11B2 is enabled by an increase in retention time of the intermediates in the active site due to higher intrinsic flexibility compared with CYP11B1 (Strushkevich et al., 2013). A reduced flexibility of CYP11B1 might impede the access of the non-natural substrate to the active site and thus matches the lower binding affinity and catalytic efficiency of CYP11B1 toward OT.

As revealed by NMR characterization of the products, both CYP11B isoforms catalyze the same reactions with OT as with their natural substrates. An 11β-hydroxylation is performed by both enzymes and an additional subsequent 18-hydroxylation and 18-oxidation by CYP11B2. To the best of our knowledge, the respective products have not been described in the literature thus far. We can hypothesize the structure of metabolites based on studies on natural substrates (Kawainoto et al., 1990; Bureik et al., 2002a; Hobler et al., 2012). CYP11B1 and CYP11B2 generate the intermediate product 2 that is hypothesized to be 18-OH-OT. Metabolite 3 can be assumed to be a 19-hydroxylated OT derivative (Schiffer et al., 2015a). OT is thus the first exogenous substrate that is described to undergo all three reactions catalyzed by CYP11B2 with the same regio- and stereoselectivity as the endogenous substrate. The CYP11B2 crystal structure in complex with DOC (Strushkevich et al., 2013) revealed that it is bound to the active site via its 3-keto and 21-hydroxy groups. The absence of a 21-hydroxy group in OT does conclusively not negatively influence the selectivity or advancement of the catalytic reaction. However, the 3keto-Δ4 motif, which was already supposed to be conserved among all CYP11B substrates using endogenous steroids (Strushkevich et al., 2013), is preserved and seems to be sufficient for proper binding. Interestingly, CYP11B2 metabolism of metandienone, which is structurally identical to OT except for the 4-chloro group, comprises only monohydroxylations in positions 11β and 18 (Parr et al., 2012). Compared with the metabolites formed from OT by CYP11B2, the differences between OT and metandienone metabolism lead to the suggestion that the C_4 substitution of OT determines the processivity of the reaction.

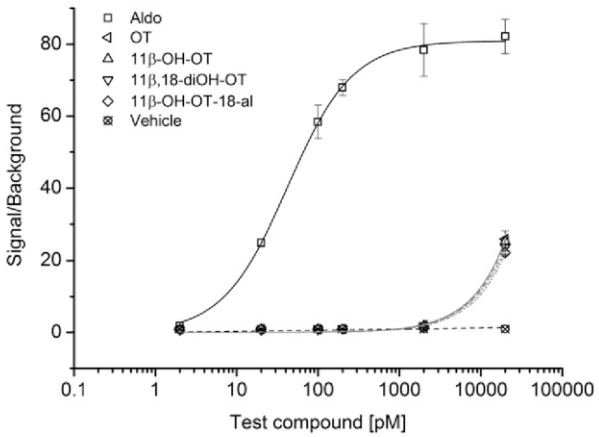

Fig. 8. Dose-response analysis of human MR activation by OT and its CYP11B-derived metabolites. Dilutions of each compound were analyzed with the INDIGO Bioscience Human Mineralocorticoid Receptor Reported Assay System following the manufacturer's protocol. The luminescence signal of MR-responsive luciferase reporter gene expression was normalized to the signal of the vehicle control and is plotted against the test compound concentration in a logarithmic scale. The assay was performed in triplicate for each concentration. The black line indicates the Hill-regression of the aldosterone-induced response (R^2 = 0.998), and the dashed line shows the vehicle background signal. Aldo, aldosterone.

It is worth mentioning that the conducted bioconversion of OT by CYP11B2 at the preparative scale represents the first application of human CYP11B2 for substrate conversion in *E. coli*, which has thus far only been used for biotechnological purposes in recombinant yeast strains (Bureik et al., 2002b). Despite working under nonoptimized conditions with protein synthesis and substrate conversion taking place in shake flasks, the volumetric productivity of OT consumption of approximately 30 mg/l per day reaches the minimum requirements for potential application in industrial pharmaceutical production (Julsing et al., 2008). CYP11B2-dependent aldosterone biosynthesis plays a crucial role in the regulation of blood pressure and related diseases (Ardhanari et al., 2015), and researchers are regaining interest in the use of selective CYP11B2 inhibitors in treatment of these diseases (Andersen, 2013; Hargovan and Ferro, 2014; Namsolleck and Unger, 2014). The presented system might alternatively serve as simple, economic prescreening for the effectiveness of potential inhibitors upon downscaling to a multiwell format.

Compared with the two CYP11B enzymes, CYP11A1 exhibits a lower efficiency of OT conversion (46 min^{-1} mM^{-1} compared with 741 min^{-1} mM^{-1} and 3338 min^{-1} mM^{-1}), which is also lower than for the natural substrate cholesterol. Bovine CYP11A1 shows an efficiency of approximately 85 min^{-1} mM^{-1} for cholesterol (Neunzig and Bernhardt, 2014; Mosa et al., 2015). The low catalytic efficiency for OT is, however, consistent with the observed activities toward endogenous steroids (Mosa et al., 2015) and the preference of CYP11A1 for longer side chains (Morisaki et al., 1980). Because of low productivity, structural assignments for the emerging OT metabolites were proposed for three of the six metabolites from MS/MS product ion mass spectra and previous studies that describe 2β-, 6β-, and 16β-hydroxylase activity of CYP11A1 for steroids (Mosa et al., 2015) (Fig. 3). Metabolite 7 shows an unmodified A/B-ring and an OH-group that can be assigned to the C- or D-ring. It is thus proposed to be 16-OH-OT. The product ion mass spectrum of metabolite 9 provides strong indications for an A-ring hydroxylation and as positions 3 and 4 are occupied, we suggest 2-OH-OT as a putative structure. Metabolite 11 seems to carry a combination of these two hydroxylations and is assumed to be 2,16-diOH-OT. 6- and 16-Hydroxylated OT species are already known from previous metabolism and excretion studies. In humans, OT is mainly transformed by reduction of the A-ring double bonds and keto group; by hydroxylation in positions 6β, 12, and 16β by CYP3A4, among others; and by rearrangement to 18-nor-17β-hydroxymethyl derivatives (Schänzer, 1996; Schänzer et al., 1996; Rendic et al., 1999; Sobolevsky and Rodchenkov, 2012). 2-OH-OT would hence be another new OT metabolite discovered in this study.

After studying the biotransformation of OT by human mitochondrial P450 enzymes, we were also interested to examine the effect of OT on the activity of these P450 enzymes toward their endogenous substrates. An inhibition of the natural function of OT-metabolizing P450 enzymes seemed likely, because both OT and natural substrates compete for binding to the active site with comparable affinities. In fact, an inhibitory effect of OT on the conversion of natural substrates was demonstrated by our in vitro data. Inhibition thereby depends on the concentration and mirrors the affinity of P450 enzymes for OT, resulting in the highest inhibitory effect on CYP11B2. Potential physiologic relevance is, however, the result of concentrations in the microenvironment of steroid-synthesizing enzymes and can hardly be assessed at this time. The cholesterol level in the inner mitochondrial membrane is controlled by StAR protein activity, which varies upon signaling via the hypothalamic-pituitary-adrenal axis and other steroidogenic stimuli. Plasma levels of the prohormone 19-norandrostenediol and its active metabolite nandrolone were reported to be in the range of several hundreds of nanograms per 100 ml after a single dose of 25–100 mg (Schrader et al., 2006), which is in excess of DOC and 11-deoxycortisol plasma levels (Mason and Fraser, 1975). Exact 19-norandrostenediol and metabolite plasma levels were, however, also dependent on the route of administration. DOC levels are generally lower than 11-deoxycortisol levels and CYP11B2 is expressed at low levels compared with other steroidogenic P450 enzymes. These facts and the observation of the strongest inhibitory effect of OT on CYP11B2 indicate that the biggest effect in vivo of OT can be expected for aldosterone synthesis from DOC. This hypothesis of a relevant inhibition of CYP11B2-dependent aldosterone synthesis by OT coincides with AAS-induced increases in DOC levels in rats, whose MR-agonist properties induce increases in blood pressure (Colby et al., 1970). However, this phenomenon has thus far only been explained on a transcriptional level (Brownie et al., 1970; Colby et al., 1970; Brownie et al., 1988; Gallant et al., 1991). Our new insights suggest additional direct effects on an enzymatic level.

The identification of metabolites and characterization of their bioactivity is a crucial step in drug design. The elucidation of the MR crystal structure with various ligands (Bledsoe et al., 2005; Li et al., 2005) displayed that a hydrogen bond network between the steroid ligand C_{11} and C_{18} positions and the ligand binding domain is crucial for its activation. The alteration of the hydrogen bond–forming properties of these positions can thus modulate the agonist activity of a steroid. We therefore tested OT and the new metabolites, which are produced by the CYP11B enzymes, for their potential to activate the MR. It turned out that OT acts only as a very weak MR agonist. The new metabolites also do not show a relevant activation of the MR, although they carry oxy-functionalization at C_{11} and C_{18}. An endocrine-disrupting potential of OT and its metabolites at the MR is thus unlikely. It can be hypothesized that OT and its metabolites do not induce all conformational changes required for activation, because they lack the C_{20}-carbonyl and C_{21}-hydroxyl functions, which interact with the receptor in presence of the native ligands (Bledsoe et al., 2005). In addition, it is assumed that the length of the ligand also determines the events leading to activation (Bledsoe et al., 2005). This assumption is consistent with the weak binding of the C_{19}-steroid 1-testosterone to the MR (Friedel et al., 2006a) and the higher affinity of tetrahydrogestrinone, which has a C_{17}-ethyl group (Friedel et al., 2006b).

In summary, these results clearly demonstrated that OT is the second xenobiotic steroid whose metabolism by steroidogenic P450 enzymes has been observed, in addition to metandienone (Parr et al., 2012). Our detailed in vitro studies hint at a potentially systematic contribution of human steroidogenic P450 enzymes to the metabolism of xenobiotics, which suggests their consideration as drug-metabolizing enzymes during drug design and toxicity evaluation. Their metabolic potential and a need for their involvement in drug testing appears especially important in the case of steroidal drugs, which are widely applied to treat a variety of anti-inflammatory and contraceptive issues as well as for disease- or cancer-conditioned (postoperative) steroid replacement purposes. Product properties might differ from those of the parental compound, and the interference with the endogenous steroid biosynthesis can cause severe adverse effects.

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Authorship Contributions

Participated in research design: Schiffer, Thevis, Bernhardt.
Conducted experiments: Schiffer, Brixius-Anderko, Zapp, Neunzig.
Performed data analysis: Schiffer, Zapp, Thevis.
Wrote or contributed to the writing of the manuscript: Schiffer, Hannemann, Thevis, Bernhardt.

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2.5 Lina Schiffer et al. (2015b)

The CYP11B subfamily- Review

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Review

The CYP11B subfamily

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ABSTRACT

The biosynthesis of steroid hormones is dependent on P450-catalyzed reactions. In mammals, cholesterol is the common precursor of all steroid hormones, and its conversion to pregnenolone is the initial and rate-limiting step in hormone biosynthesis in steroidogenic tissues such as gonads and adrenal glands. The production of glucocorticoids and mineralocorticoids takes place in the adrenal gland and the final steps are catalyzed by 2 mitochondrial cytochromes P450, CYP11B1 (11β-hydroxylase or P45011β) and CYP11B2 (aldosterone synthase or P450aldo). The occurrence and development of these 2 enzymes in different species, their contribution to the biosynthesis of steroid hormones as well as their regulation at different levels (gene expression, cellular regulation, regulation on the level of proteins) is the topic of this chapter.

Contents

1. Diversity on organismic level: function, expression and evolution of the CYP11B subfamily

CYP11B1 and CYP11B2 are members of the cytochrome P450 (P450) superfamily. P450s belong to a highly conserved class of enzymes that occur in every domain of life. They catalyze various reactions which are indispensable for many species like detoxification, defense and biosynthesis of endogenous compounds such

Abbreviations: A, aldosterone; B, corticosterone; DOC, 11-deoxycorticosterone; E, cortisone; F, cortisol; S, 11-deoxycortisol.

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as steroid hormones. As external monooxygenases they are able to utilize the activation of molecular oxygen for substrate hydroxylation. But their range of possible reactions is much higher comprising almost 30 different reaction types [1–3]. Despite their highly conserved structural properties, the amino acid composition differs from enzyme to enzyme which contributes to a high diversity among the superfamily, consisting of more than 21,000 genes (http://drnelson.uthsc.edu/CytochromeP450.html). An outstanding example for enzyme diversity in contempt of their evolutionary conserved function and highly similar structure is given by enzymes belonging to the CYP11B subfamily. These proteins are located in the inner mitochondrial membrane where they are supplied with electrons from NADPH via 2 electron transfer proteins, the adrenodoxin reductase (AdR) and adrenodoxin (Adx).

In the following subchapters, the CYP11B interspecies diversity, their role and regulation within mammalian endocrine systems as well as their divergence during evolution and species dependent endocrine functions will be discussed.

1.1. Mammalian CYP11B isoforms – evolution, interspecies diversity and expression pattern

Steroid hormone synthesizing P450s like the CYP11B subfamily members seem to originate from early detoxifying variants and there is evidence for a co-evolutionary process with their particular steroid substrates [4]. Recent phylogenetic studies revealed a common ancestor of mitochondrial cytochromes P450 which evolved by duplication events into the enzymes of the CYP11A and the CYP11B subfamily [5]. In human, 2 CYP11B isoforms exist which are involved in the biosynthesis of 2 of the most important steroid hormones (steroids are abbreviated as defined on page 1): CYP11B1 (11β-hydroxylase) catalyzes the final step of F synthesis by a one-step reaction from S, whereas A is synthesized via 3 steps by CYP11B2 (aldosterone synthase) from DOC [6–8]. The distinct reactions will be discussed more detailed later in this review. Human CYP11B enzymes show an amino acid similarity of 93% and their genes are tandemly arranged on chromosome 8 with a distance of 40 kbp. Evidently, the possession of 2 distinct enzymes is restricted to mammals, whereby the CYP11B2 homologs show a higher similarity towards each other than CYP11B1 homologs do, which proofs the evolution of CYP11B2 by a gene duplication of CYP11B1 [9]. Comparably to humans, two functionally distinct CYP11B isoforms can be found in mouse [10], guinea pig [9,11], baboon [12,13] and hamster [14]. Rats exhibit even 3 enzyme variants, CYP11B1, CYP11B2 and CYP11B3, whereupon the CYP11B3 isoform is only expressed for a few days during postnatal development [15]. In rat, B is the main glucocorticoid. Interestingly, cattle (*Bos taurus)* possesses 2 variants of a CYP11B enzyme, commonly assigned as CYP11B1, that perform both F [16] and A [16–21] biosynthesis. Cattle CYP11B1 has been extensively studied and much of our present knowledge on the CYP11B subfamily has been derived from this enzyme. In total, the bovine genome encodes 5 CYP11B1 genes [22]. Three of these represent pseudogenes [22,23]. The 2 functional genes encode 2 distinct, but very similar CYP11B1 variants with only slight functional varieties [18,24,25]. Moreover, structural diversity of bovine CYP11B1 is enhanced by the occurrence of polymorphisms [26,27]. Gluco- and mineralocorticoid synthesis is also catalyzed by a single enzyme in porcine [19], frog [28] and sheep [29].

CYP11B enzymes have to be strictly regulated on transcriptional, translational and post-translational level because of the higher demand of glucocorticoids compared to mineralocorticoids (Fig. 1). Therefore, a complex network of mechanisms exists starting with different gene expression patterns in adrenal tissues. In human as well as in rat it was demonstrated that the expression of

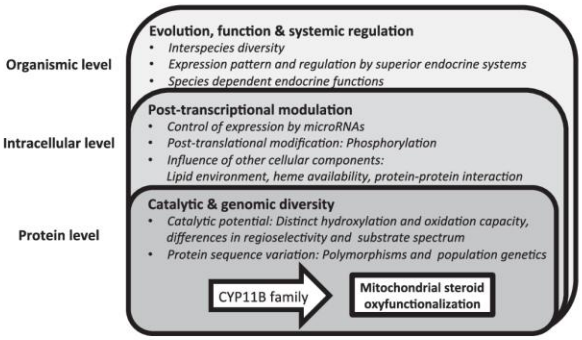

Fig. 1. Levels of CYP11B diversity discussed in this review.

CYP11B1 and CYP11B2 in the adrenal glands is spatially separated. While expression of CYP11B1 takes place in the *zona reticularis/fasciculata,* CYP11B2 expression and A synthesis are restricted to the *zona glomerulosa* [30–34]. F production by CYP11B1 is, however, limited to the *zona fasciculata* because of a 3β-hydroxysteroid dehydrogenase deficiency in the *zona reticularis* [35]. Recent studies revealed that in human adults CYP11B2 expression and CYP11B2 catalyzed A production can also occur in so-called subcapsular aldosterone-producing cell clusters (APCCs) of non-pathologic origin whose functional role and formation are not clear yet [36,37]. In cattle, A and F production are also limited to the respective adrenal zone, but due to the fact that CYP11B1 is localized in all 3 zones of the adrenal cortex [38] and synthesizes both steroids, this zonation cannot be explained by a different expression pattern and needs to be further investigated. Since bovine CYP11B1 is able under *in vitro* conditions to produce F and A, factors that suppress A formation in the *zona glomerulosa* need to be identified. However, until now, all attempts to identify such specific factors were unsuccessful. Although it was shown that angiotensin II could support A production in fetal bovine adrenal cells, an analysis of the promoter regions did not give an insight into zone-dependent A and F formation in cattle [32,39]. Current studies focusing on epigenetic aspects of CYP11B expression support the idea that a gene silencing of the particular CYP11B-variant by a hypermethylation of the gene in the respective tissue could lead to adrenal zonation [40]. Therefore, epigenetics is a promising approach for further elucidation of adrenal zonation and CYP11B regulation mechanisms, especially in case of cattle.

Another important aspect is the expression of the CYP11B enzymes in extra-adrenal tissues. There is evidence for an expression in rat and human brain but the physiological function remains unclear [41,42]. The CYP11B2 expression in heart is controversial [43–45]. It is however a fact that A can lead to cardiac fibrosis and heart insufficiency [46,47].

The distinctly controlled and induced production of gluco- and mineralocorticoids is a result of a highly sensible interaction between crucial components of the hypothalamic–pituitary–adrenal axis (HPA) and the renin–angiotensin-system (RAS). CYP11B2 expression and A secretion are mainly controlled by angiotensin II and potassium which cause an increase of the cellular calcium level, leading to the activation of calmodulin and of calmodulin-dependent kinases, subsequently. It is assumed that the activated kinases phosphorylate both activating transcription factors and members of the CRE-binding protein (CREB, cAMP-regulated binding protein) family which bind to 5′ flanking promoter regions of the CYP11B2 gene and trigger gene transcription in the *zona glomerulosa* [32,48,49]. Studies revealed that the adrenocorticotropic hormone (ACTH) can also influence

CYP11B2 expression. However, the effect distinguishes itself by a short-term activating and long-term suppressing effect on A secretion, which is elaborated in detail in a recent publication [50]. In the *zona fasciculata,* CYP11B1 expression and F (or B in case of rat) secretion are controlled by ACTH and a cAMP regulated signaling pathway, which also involves the CREB protein family. Rainey and Omura described in detail the different regulatory mechanisms of CYP11B1 and CYP11B2 gene transcription [32,51]. Gene regulation of the respective isoforms of CYP11B takes place by distinct mechanisms to ensure the right ratio of gluco- to mineralocorticoids. Considering the highly conserved core promoter regions of each enzyme across species, there are significant differences in the 5′ upstream region between CYP11B1 and CYP11B2 which gives an explanation for the specific transcriptional regulation mechanisms [32]. Recently, also the role of transposable elements which could contribute to intra- and interspecies evolutionary diversification of the CYP11B enzymes was described. It was shown that Alu and L1 elements located 5′ upstream of the core promoter sequence can modulate the transcription of both genes while Alu has an enhancing effect on both genes and L1 elements can block the effect of Alu on CYP11B1 transcription [52].

In the next section adrenal CYP11B expression will be discussed in the context of complex endocrine systems in mammals.

1.2. The role of the endocrine system for CYP11B expression

The high conservation of CYP11B proteins can be explained by the outstanding role of their end products for the development of terrestrial life. Throughout all evolutionary processes crucial metabolic and physiological pathways have been highly conserved despite divergence events and subsequent species diversity. One essential pathway for survival is the HPA axis which occurs exclusively in vertebrate species. This complex network consists of different peptides, receptors and steroid hormones and its action is highly diverse. Various stimuli like stress, illness or the circadian rhythm activate the HPA via the release of CRH (corticotropin-releasing hormone) in the hypothalamus which leads to a stimulation of the anterior pituitary gland and a subsequent release of ACTH [53]. ACTH binds its receptor in the adrenal glands and induces CYP11B1 expression and, therefore, the secretion of F. F subsequently evokes systemic stress responses through glucocorticoid receptor interaction and activation of various targets involved in energy mobilization, glucose homeostasis, immune response and behavior [54–57]. Besides, F is involved in ontogenetic processes, such as sex differentiation and the development from embryonic to adult organisms [58] and it can also regulate, in coexistence with the appropriate receptor, the mineral balance in organisms which are not able to produce A like some fish species. This highlights the promiscuity of corticosteroid action within diverse metabolic and physiological processes [59]. A feedback regulatory effect of F represses CRH release and ensures the fine-tuning of HPA activity by avoiding an excess of circulating glucocorticoids [60,61].

A dysregulation of this accurately synchronized network can lead to physical disorders, but also to mental illness, such as depressions, an exploding social phenomenon in our times [62,63]. Protein constituents of the HPA like nuclear receptors or peptide hormones, e.g., ACTH, are highly conserved within metazoa and seem to have evolved from the same ancestors emphasizing their crucial role in vertebrate development and terrestrial life [64]. An exciting observation occurring upon HPA evolution is the co-evolution of nuclear receptors and the corresponding steroids which is leading to a refinement of the various physiological responses [4]. Although the classical vertebrate HPA is not found in invertebrates like ascidiens because of an early divergence, many HPA components and homologous synthesis islands occur individually which hints at a possible bridging between the vertebrate and invertebrate endocrine systems [65]. This phenomenon can be further elucidated with the help of suitable model organisms like zebra fish [66].

Higher vertebrates acquired the capability to synthesize A which regulates the salt and water homeostasis and allowed the evolvement of terrestrial life by sophisticated water reabsorption strategies. In contrast to the biosynthesis of F, A production and thus water reabsorption, osmotic regulation and blood pressure are mainly regulated by the RAS which controls adrenocortical CYP11B2 expression [67]. An overproduction of A is associated with hypertension and accordingly, CYP11B2 is a promising target in drug development, as selective inhibitors could decrease A level and blood pressure [68–70]. The independent regulation of the CYP11B enzymes by different superior endocrine systems guarantees their expression in accordance with the physiological need for their respective products, F and A.

1.3. Low vertebrate CYP11B isoforms – evolution and species dependent endocrine functions

CYP11B orthologous genes have been identified in over 25 species ranging from teleosts to mammals (http://www.ncbi.nlm.nih.gov/gene/?Term=ortholog_gene_1584 [group]). Because of the high conservation of stress axis mechanisms during the evolution of vertebrates, great efforts were made over the last years to identify and characterize conserved components including enzymes of the CYP11B subfamily in lower vertebrates, with focus on fish, which represent the lowest species among vertebrates. Several new members of the CYP11B subfamily (CYP11B isoforms from fish are also termed CYP11C) were discovered and are reviewed in [71]. While in mammals the synthesis of F and A represents the main function of CYP11B enzymes, the synthesis of 11-oxygenated androgens is an additional crucial function of CYP11B enzymes in fish, which lack A, except for a few species, and use F as main gluco- and mineralocorticoid, which is produced in the head kidney [72]. Fish CYP11B enzymes also catalyze the 11β-hydroxylation of testosterone to 11β-hydroxytestosterone in gonads. 11β-hydroxytestosterone is the direct precursor of the testicular androgen 11-ketotestosterone [73], which has been described for a long time as sex determining male hormone in fish [73]. More recent studies could now establish the direct relationship between CYP11B expression and testicular development and differentiation in sea bass [74] and pejerry [75], male morph-specification in vocal fish [76], spermatogenesis in *rainbow trout* and *nile tilapia* [77,78] and sex changes to a male phenotype in hermaphroditic teleosts [77,79].

The identification of new enzyme subfamily members from evolutionary lower species contributes to our understanding of evolution and divergence within the group of CYP11B enzymes. There is evidence from gene cluster analysis that CYP11B1 seems to have evolved from fish CYP11C1 [5]. CYP11C1 of zebra fish is catalyzing the formation of the main glucocorticoid F. Therefore, zebra fish represents a promising and genetically amenable model organism with conserved endocrine mechanisms compared to higher vertebrates. A further potential key player for the elucidation of CYP11B evolution might be the functionally not identified CYP11-like gene sequence from lancelet, which represents an important transitional fossil at the border between invertebrates and vertebrates [80].

In addition to interspecies diversity and functional diversity induced by the endocrine system, CYP11B systems were discovered to be modulated by several factors on intracellular level. Furthermore, diversity and promiscuity can be found on protein level represented in variations of the catalytic potential and

protein sequence variation with distinct population genetics. These two levels (Fig. 1) will be discussed in the next chapters.

2. Modulation of CYP11B systems on intracellular level

2.1. MicroRNAs in post-transcriptional regulation

The transcriptional control mechanisms by the CYP11B promoters [32,48,52,81,82] and the influence of polymorphisms in the CYP11B genes [83–86] on transcription have been described by several studies. In addition to the topic of transcriptional regulation, which is not the focus of this review, the increasing knowledge about the role of microRNAs (miRNAs) opens up new perspectives on a further regulation level for the CYP11B1 and CYP11B2 genes as well as for the derived proteins and steroid hormone production.

Post-transcriptional regulation by miRNAs, which have emerged as key regulatory molecules that control ~30% of all mammalian genes [87], is a hitherto less studied issue in the field of steroid biosynthesis. MiRNAs are endogenous single-stranded noncoding RNA molecules of ~22 nucleotides that can play an important regulatory role by targeting specific sequences on mRNAs of protein-coding genes to direct their post-transcriptional repression [88]. So far, the few studies investigating the role of miRNAs in steroid hormone biosynthesis documented an influence on adrenal cell development and survival [89], dysregulation in adrenal carcinomas [90] and regulation of A production [91]. Recently, the role of miRNAs was investigated with particular focus on the genes of the human CYP11B subfamily [92]. It was shown that the expression of CYP11B1 and CYP11B2 in a human adrenocortical cell line depends on the presence of *Dicer1*, a key enzyme in miRNA maturation, and on the function of microRNA miR-24, for which binding sites in the 3′ untranslated region of CYP11B1 and CYP11B2 mRNAs were identified. The miR-24 regulates both genes in a canonical miRNA manner and seems to mediate their repression to a similar degree.

With the knowledge of this new post-transcriptional regulation level, it has to be considered whether siRNAs can be applied for the silencing of e.g., CYP11B2, which might be a novel approach to treat metabolic diseases like hyperaldosteronism, aldosterone caused hypertension and congestive heart failure.

2.2. The role of phosphorylation in regulating CYP11B-dependent activities

Although a wealth of interesting and promising results on the regulation of mitochondrial steroid hydroxylase systems on the transcriptional level have been obtained, far less attention has been paid to investigate post-translational modifications of the CYP11B electron-transfer systems, consisting of AdR, Adx and the respective P450 (Fig. 2). In this context, studies concentrated on protein phosphorylation, which is likely to alter the steric, electrostatic, and hydrogen bonding properties of the proteins. This is of particular relevance for cytochrome P450 systems because the introduction of a charged phosphate group might significantly influence the redox properties of the involved proteins or their binding behavior during the redox complex association and dissociation.

Early investigations of the phosphorylation state of rat adrenal mitochondrial proteins indicated that ACTH stimulated both phosphorylation and dephosphorylation of adrenal proteins and increased the B production 6-fold [93–95]. However, a specific target could not be identified in these early studies. The question whether mitochondrial cytochromes P450 represent regulation targets for phosphorylation or not continued to remain unclear in the following studies. On the one hand, experiments provided

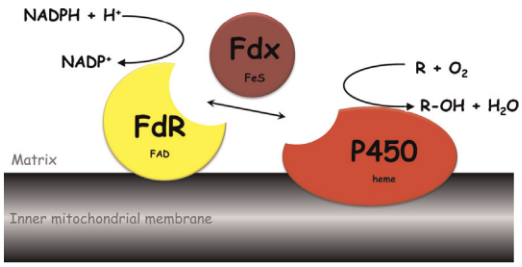

Fig. 2. The mitochondrial electron transfer system. Electrons are delivered along the inner mitochondrial membrane from NADPH via the redox proteins AdR, a membrane associated ferredoxin reductase (FdR), and the soluble ferredoxin (Fdx) Adx to members of the membrane incorporated CYP11B subfamily. The redox centers, flavin adenine dinucleotide (FAD), iron-sulfur cluster ([2Fe2S]) and Fe-protoporphyrin IX (heme) are indicated.

evidence that a crude preparation of cytochrome P450 from bovine corpus luteum mitochondria was phosphorylated *in vitro* in a cAMP-dependent reaction and consequently stimulated steroidogenesis [96], on the other hand, studies performed with preparations from rat adrenals could not confirm the stimulating effect [97]. In contrast to that, direct evidence has been provided that CYP11B1, purified from bovine adrenal cortex, can be phosphorylated using the cAMP-dependent protein kinase subunit C [98]. This phosphorylation increased the CYP11B1-Adx affinity, which was discussed to be of potential physiological importance for the steroidogenesis under Adx limited conditions.

Beside the CYP11B enzymes, the electron shuttle proteins of P450 systems attracted attention as phosphorylation target. It was demonstrated that renodoxin, the renal ferredoxin (analogous to Adx) [99], can be phosphorylated and following studies revealed that renodoxin and its phosphorylated form have a differential regulatory effect on the activity of the 1α- and 24-hydroxylases, CYP27B1 and CYP24A1, respectively [100,101]. In case of the bovine adrenal ferredoxin, *in vitro* phosphorylation experiments documented that the protein can be selectively modified at residue Thr-71 using the protein kinase CK2 [102] and at residue Ser-88[1] using the cAMP-dependent protein kinase (PKA) [103]. Adx phosphorylated by PKA increased the activity of reconstituted CYP11A1 and CYP11B1 systems, whereas Adx phosphorylated at residue Thr-71 increased only substrate conversions catalyzed by CYP11A1. Since both phosphorylation positions are located within the redox partner interaction domain of Adx it was supposed that steroid hydroxylase activity can be modulated on the post-translational level by influencing the protein interactions between the redox-partners of the P450 system [102,104]. Due to only very recent availability of human CYP11B1 and CYP11B2 for *in vitro* studies [105,106], corresponding studies on the effect of Adx phosphorylation on their activities have not been performed so far.

However, in order to establish the significance of such post-translational modifications in cell cultures kinase inhibitor studies were performed in V79 lung fibroblast cells [107], which expressed human CYP11B1 and CYP11B2 constitutively. Applications of kinase inhibitors indicated differential changes of the steroid hydroxylation activity of CYP11B1 and CYP11B2 depending on the inhibitor used. This supported the hypothesis that CYP11B enzyme systems are specifically regulated by phosphorylation processes and points to a modulation of the CYP11B activity by phosphorylation *in vivo*. In order to prove this hypothesis further studies are greatly needed to verify the phosphorylation targets, the natural

[1] Later studies revealed that PKA phosphorylation does not take place in this position (Bernhardt unpublished results).

regulatory mechanisms and the physiological meaning for the synthesis of F and A.

2.3. Modulation of CYP11B1 and CYP11B2 activities by cellular components

After their synthesis, CYP11B enzymes are transported to the inner membrane of the mitochondria. Once integrated in the phospholipid bilayer, the membrane and its associated components provide the environment to maintain the full enzyme function and may influence the catalytic and physico–chemical properties of these enzymes [108] (Fig. 2). One factor that is influencing enzyme activity on this level is the presence and composition of phospholipids. It was described that phospholipids extracted from adrenocortical mitochondria remarkably enhanced the CYP11B activity in reconstituted systems [109]. This effect was analyzed in more detail in studies indicating that the stimulation is depending on the presence of neutral or acidic phospholipids and on the type of fatty acyl substituents of the lipids [110,111].

A second CYP11B activity influencing factor is the availability of heme, which is essentially required for the assembly of a functional cytochrome P450. This is in agreement with the finding that activation of adrenal cytochromes in the adrenocortical cell line H295R by ACTH induces simultaneously the heme biosynthesis [112]. It was also shown that the administration of hemin stimulated B and A production in rat adrenal homogenates and in primary cultures of the calf adrenal *zona glomerulosa* in a dose-dependent fashion, whereas 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC), an inhibitor of heme synthesis, partially blocked ACTH-mediated steroid hormone synthesis [113,114]. These studies indicate that an influence on the activity of CYP11B enzymes can also take place on the level of protein folding depending on the synthesis and proper incorporation of heme into CYP11B enzymes.

Other factors modulate the activity of CYP11B enzymes by affecting redox partner-interactions. Calmodulin and another mitochondrial P450, CYP11A1, were described to influence the CYP11B affinity to electron transport partners as well as substrates. The adrenocortical form of calmodulin, a small, versatile and ubiquitous intracellular trigger protein, was detected to interact with bovine CYP11B1 in the presence of Adx modulating the activity of A synthesis [115]. The study described that the calmodulin effect is Ca^{2+} dependent and led to a decreased rate of A production, while it increased the rate of 18OH-B formation. Another protein that influences CYP11B activity is CYP11A1, the cholesterol side chain cleavage enzyme, which was intensively investigated with respect to its modulation of CYP11B enzymes. On the one hand, competition for reducing equivalents was observed between rat CYP11A1 and CYP11B1 *in vitro* [116] as well as in COS-1 cell cultures expressing human and bovine enzymes [117] which results in a decrease of F and A synthesis under conditions with limited resources of reducing equivalents. On the other hand, results indicated that bovine CYP11A1 interacted specifically with CYP11B1 and stimulated its 11β-hydroxylation activity while it decreased 18OH-B and A formation in reconstituted liposomal membranes [118,119] and COS-1 cells [117]. It has to be mentioned that an allosteric influence of CYP11A1 on the product pattern of CYP11B enzymes seems to be species-dependent, because such an effect has not been observed for the human enzymes in COS-1 cells [117].

In addition to CYP11A1, the efficiency of interaction with the redox partner Adx is also of high impact for CYP11B activities, although the role of Adx is often underestimated [120]. Adx as essential part of the NADPH-dependent redox system, delivers electrons to all mitochondrial cytochromes P450 (Fig. 2). It has been investigated with regard to both binding and electron transfer to various mitochondrial cytochromes P450 as well as to its influence on substrate binding and activity modulation. Several defined residues and protein domains were shown to be necessary to form a functional Adx-P450 complex, whereby overlapping but not identical binding sites for different P450s (CYP11B1, CYP11A1) have been identified using bovine proteins [121,122]. Moreover, it has been observed that enzyme activities were low in recombinant systems using COS-1, V79 cells or *fission yeast* if only the cytochrome P450 was expressed using the autologous redox partners and can be significantly stimulated upon cotransfection of an Adx gene [26,123–126]. This shows the importance of the Adx concentration and of efficient redox partner interactions with mitochondrial P450s for their activity. Interestingly, a recently performed study using reconstituted systems containing purified human CYP11B proteins only confirmed an Adx-dependent stimulation for CYP11B2 but not CYP11B1 [127]. In contrast, a stimulatory effect was caused by N- and C-terminally truncated forms of Adx with respect to B formation by bovine CYP11B1 [128]. In addition, a shift of the product equilibrium towards 18OH-B and A was observed in reactions of bovine CYP11B1 and human CYP11B2 studied in COS-1 cells when using Adx species displaying a higher efficiency of electron transfer to bovine CYP11B1 and human CYP11B2 [129]. This observation is consistent with Biacore and stopped-flow measurements, which indicated that complex rearrangements or conformational gating, induced by Adx might be necessary before an efficient electron transfer can take place in the complex with human CYP11B1 [106]. These results, together with the observation that a proteolytic digestion of the C-terminal part of Adx in the bovine adrenal gland produced several truncated Adx forms [130–132], led to the assumption that Adx expression level in general and its potential proteolytic modification in particular may regulate steroid hormone biosynthesis [133].

3. Diversity of CYP11B enzymes on protein level

3.1. Variation of catalytic potential and product spectrum between CYP11B isoforms

CYP11B isozymes are well known to be responsible for the final steps in the biosynthesis of gluco- and mineralocorticoids by catalyzing the formation of F and A from S and DOC, which are produced from their respective precursors by CYP21A2. However, the spectrum of substrates that are converted by CYP11B isozymes and the distinct functions fulfilled by the different enzymes from various organisms demonstrate a clear diversity between isozymes of the CYP11B subfamily. The on-going exploitation of their substrate spectrum ascribes them additional roles in biotransformations other than F and A synthesis. These aspects of functional diversity and promiscuity will be elucidated in the following section by taking examples of the CYP11B isozymes from bovine, human and rat as organisms with 1, 2 and 3 functional CYP11B enzymes, respectively. Fig. 3 sums up the respective activities of the discussed isozymes in transformations of the A and F precursors DOC and S.

3.1.1. Rat CYP11B enzymes

Rattus norvegicus expresses 3 different CYP11B enzymes [134,135] that have evolved with clearly defined functions in steroid hydroxylation and oxidation within the synthesis of mineralo- and glucocorticoids.

The predominant function of the first rat isozyme, CYP11B1, is the 11β-hydroxylation of DOC and S to B and F, respectively [7,136–139]. One should remember at this point that in rat B represents the major glucocorticoid, and not F like in cattle or human [30]. Besides the 11β-hydroxylation, CYP11B1 displays minor activities as 18- and 19-hydroxylase. The respective

products occur as side-products in the conversion of DOC [7,136–138], while 18OH-S is formed as a side product from S [139]. Additionally, the products of the 11β-hydroxylation, B and F, can further serve as substrates for the 18-hydroxylase reaction yielding 18OH-B [137,138] and 18OH-F [139], respectively. Comparable to the human CYP11B1, which will be discussed later in this section, the rat isoform is not capable of performing additional reactions on 18OH-DOC [137]. Thus, for the synthesis of 18OH-B by CYP11B1, the 11β-hydroxylation seems to be required to precede the hydroxylation in position 18. In addition to its functions as hydroxylase, rat CYP11B1 is able to perform oxidase reactions toward position 11 and 19. 19OH-DOC, which occurs as side-product during the conversion of DOC, can be oxidized to the 19-oxo product [137,138] and F, as the 11β-

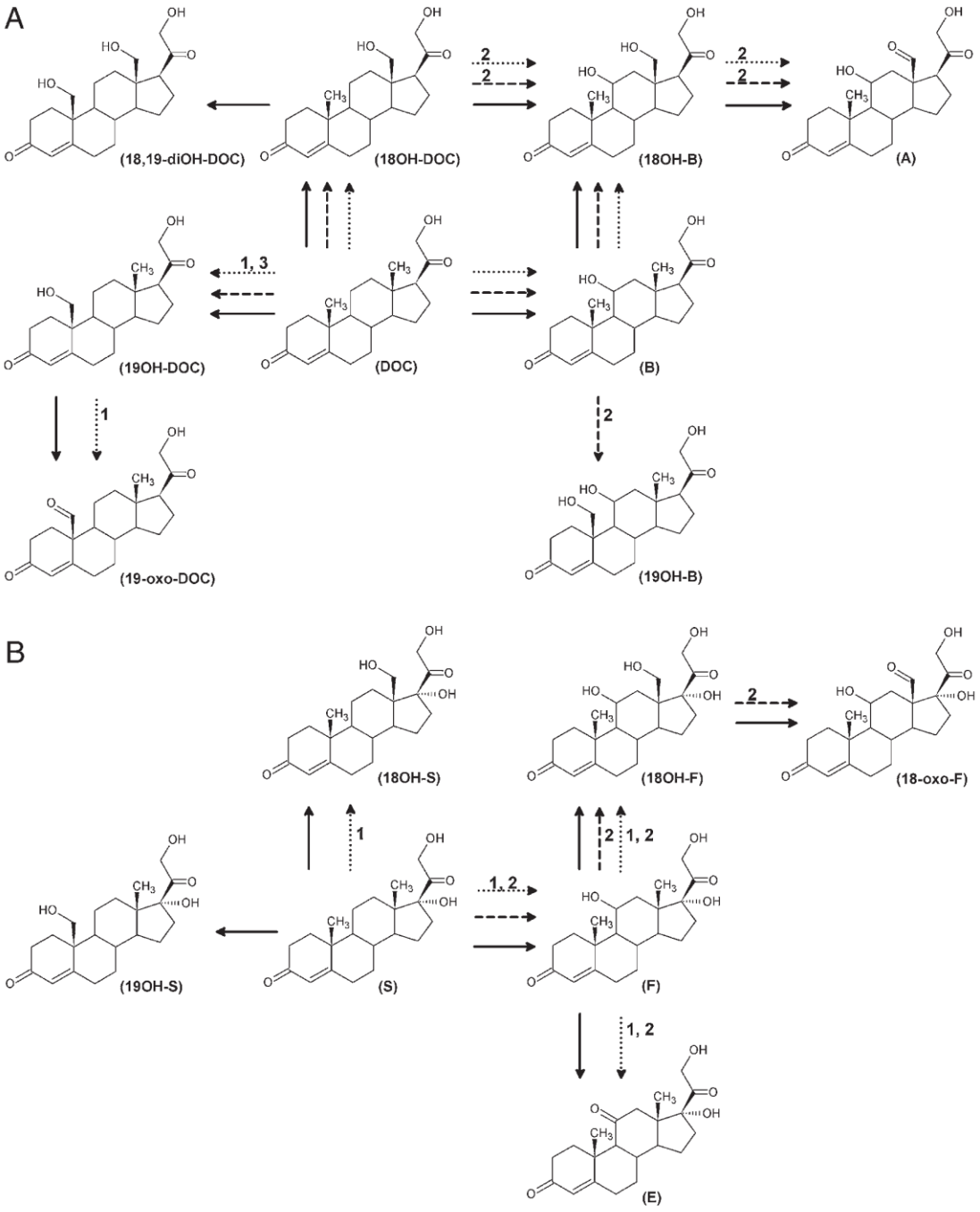

Fig. 3. Reactions catalyzed by different CYP11B isozymes from rat (dotted arrows), bovine (solid arrows) and human (dashed arrows) catalyzed from DOC (A) and S (B). For human and rat the number next to the respective arrow represents the responsible isozyme, if the reaction is not performed by all isozymes of the organism. Steroids are abbreviated as defined on page 1.

hydroxylated product of S, can subsequently be oxidized in small quantities in position 11 leading on to E [139]. Thus, rat CYP11B1 can be described as an 11β-hydroxylase with slight side activities as 18- and 19-hydroxylase as well as 11- and 19-oxidase, but it lacks an 18-oxidase function and consequently the capability of A formation.

This reaction is carried out in rat by CYP11B2, which is the only rat CYP11B isozyme that combines 11β- and 18-hydroxylase activities with an 18-oxidase activity. It forms A from DOC via the intermediates B and 18OH-B along with the formation of 18OH-DOC as side product [140]. *In vitro* activity studies indicate the existence of a distinct chronological pathway of intermediate formation, which is required for A formation by rat CYP11B2: if 18OH-DOC is used as a substrate, it is converted to 18OH-B [137,138], which is contrary to rat CYP11B1 but in agreement with the results for human CYP11B2 [127,141]. However, no consecutive oxidation to A by CYP11B2 can be observed under these conditions. This gives rise to the hypothesis, that A synthesis by rat CYP11B2 demands processive 18-hydroxylation followed by 18-oxidation. Considering S transformation by rat CYP11B2, the same products as for the CYP11B1 isozyme (F, E and 18OH-F) are formed except for 18OH-S [139]. Although no information about the involvement of CYP11B2 in 18-oxo-F synthesis is available so far, its participation in this reaction is very likely as the formation of this steroid by the rat adrenal has been observed [142].

The third rat isozyme, CYP11B3, is a pure hydroxylase without any oxidase activity. It converts DOC to B and 18OH-DOC [15,143] as well as B to 18OH-B [15]. However, in doing so the ratio of 18OH-DOC formation to B formation reveals a clear preference for 18- over 11β-hydroxylation [143], which distinguishes rat CYP11B3 from the CYP11B1 isozyme. Summarizing, it can be stated that in rat the 3 different CYP11B isozymes have distinct preferences for hydroxylation and oxidation although they display overlapping activities.

3.1.2. The bovine CYP11B1 enzyme

In *Bos taurus*, a single CYP11B enzyme, termed CYP11B1, was described to integrate an efficient 11β-hydroxylase activity with hydroxylation and oxidation activity towards position 18. It performs both F [16] as well as A [16–21] biosynthesis from S and DOC, respectively. However, more recent studies on bovine CYP11B1 activity including the purification of CYP11B1 from bovine adrenocortical mitochondria [18] and systematic analysis of cDNA libraries of the bovine adrenal gland [24,25] revealed 2 distinct variants of bovine CYP11B1 and 3 pseudogenes. Although the enzymatic characterization of the 2 variants after purification from the adrenal cortex [18] or in recombinant COS-7 cells [25] revealed slight differences in A and 18OH-B formation, both forms catalyze the same reactions and variations in activity are not necessarily considered in other studies. Most of the investigations were carried out with simple protein preparations from bovine adrenocortical mitochondria during the early stages of research on mammalian mitochondrial P450 systems.

Besides the efficient production of F and A, bovine CYP11B1 demonstrates an immense versatility in additional steroid oxyfunctionalization. Comparable to A synthesis, F can also undergo CYP11B1-catalyzed 18-hydroxylation and -oxidation, but to a lesser extent than B [144]. As CYP11B1 also functions as 19-hydroxylase, A and F production is accompanied by the formation of 18- and 19-hydroxy derivatives of the precursors DOC and S [16,21,145], but 11β-hydroxylation is significantly preferred over hydroxylation at positions 18 or 19 [16]. 18OH-DOC can further be converted by bovine CYP11B1 to 18OH-B or 18,19-dihydroxy-DOC by respective hydroxylation at positions 11β or 19 [146,147].

Besides the 18-oxidase activity that enables A formation, the enzyme also owns an oxidase activity towards positions 11 and 19 and can oxidize F to E as well as 19OH-DOC [21] and 19OH-androstenedione [148] to the corresponding 19-oxo products. 19-Oxo-androstenedione can consecutively be transformed to estrone and 19-norandrostenedione [148–150], which ascribes bovine CYP11B1 an additional aromatase and a nonaromatizing 10-demethylase function. Moreover, androgens like testosterone and androstenedione can be hydroxylated in position 11β and 19 [151]. Another class of steroids, which are subject to transformation by bovine CYP11B1, are 19-norsteroids. 19-nortestosterone has been shown to be converted into 2 products [152] and 19-norandrostenedione [150] is hydroxylated in positions 11β, 18 and 6. In summary, bovine CYP11B1 can be regarded as a highly promiscuous CYP11B isoform as it accepts a broad range of substrates including C21 steroids with and without a hydroxyl group in position 17 as well as C19 and C18 steroids. It is a multifunctional enzyme with 11β, 18- and 19-hydroxylase activity as well as oxidase activity towards all these positions. Additionally, aromatizing and non-aromatizing 10-demethylase reactions are carried out [148–150].

3.1.3. Human CYP11B enzymes

In contrast to cattle, human possess 2 CYP11B isozymes with distinct functions in the synthesis of corticosteroids. The major function of human CYP11B1 (11β-hydroxylase) is the 11β-hydroxylation of corticosteroids and thereby it is primarily responsible for the formation of F from S [108,127,133,153,154]. However, DOC also represents a good substrate for this reaction [127,153–155] but S is the preferred substrate with regard to catalytic efficiency [106,127]. The respective products of this reaction, F and B, can then be hydroxylated by CYP11B1 at position 18 yielding 18OH-F [127,154] and 18OH-B [106,127,154,155]. 18OH-B formation by CYP11B1 is in agreement with the activity of rat CYP11B1, but in contrast to the CYP11B1 isozyme from guinea pig, which is not capable of performing any further reaction on B [11]. Moreover, 18OH-DOC and 19OH-DOC are formed as side-products during the conversion of DOC by CYP11B1 [127,155]. Human CYP11B1 is thus a steroid 11β, 18- and 19-hydroxylase with the following regioselectivity: 11β >> 18 > 19.

While CYP11B1 owns no oxidase activity, human CYP11B2, is capable of carrying out an oxidase reaction towards position 18 and is responsible for the production of the mineralocorticoid A from DOC, via B and 18OH-B [127,153,154,156]. A constant but low A production is ensured by a low processivity with high intermediate release [105]. Besides this additional function, CYP11B2 also differs from CYP11B1 regarding substrate specificity, efficiency and selectivity of its hydroxylase activities. It hydroxylates S and DOC in position 11β, but less efficient than CYP11B1 [127,156]. In guinea pig, the catalytic efficiency of 11β-hydroxylation is distributed inversely and CYP11B2 has been described to convert S, DOC and androstenedione more efficiently compared with CYP11B1 [9]. As for human CYP11B1, 18OH-DOC and 19OH-DOC appear as side-products in the conversion of DOC by human CYP11B2 [105,127,156]. In addition, 2 further monohydroxylated DOC-products are formed, which have not been identified so far [105]. These results are in good agreement with the binding mode of DOC in the active site of CYP11B2, which was obtained by the recent resolution of the crystal structure of this complex [127]. It shows a rather loose coordination of DOC in the hydrophobic cavity of the active site via a hydrogen bond formed by the C21-hydroxyl group and a potential hydrogen bond formed by the 3-keto group. Hydrophobic interactions with aromatic residues along the I-helix guarantee binding from the α-face and render C11, C18 and C19 accessible for hydroxylation over the heme. Although C19 is the closest to the heme iron, C11 represents the position of steric and energetically most favorable hydroxylation [105,127], which is reflected in the corresponding catalytic efficiencies.

The results of different *in vitro* studies employing the intermediates of A formation as substrates give insight into the sequential reaction mechanism, that needs to be carried out for A formation. If 18OH-DOC, which emerges as minor product in DOC conversion, is applied, it can further be converted to 18OH-B by CYP11B2 [127,141], which represents a common feature with bovine CYP11B1. This reaction is not supported by human CYP11B1 [127,141]. Thus, CYP11B2 can form 18OH-B via 2 different pathways carrying out chronology-independent 11β and 18-hydroxylation, while CYP11B1 requires 11β-hydroxylation as the first step. However, A formation from 18OH-DOC via 18OH-B is not likely to be relevant in man [141]. When B is available, CYP11B2 produces 18OH-B and subsequently A as well as 19OH-B as a side-product [105]. The 19-hydroxylase activity of CYP11B1 is, in contrast, restricted to DOC. The deployment of 18OH-B as substrate in a reconstituted *in vitro* system with a concentration of 400 μM does not result in the formation of A and titration of CYP11B2 with increasing 18OH-B concentrations up to 1 mM does not induce a high spin shift of the heme iron [105]. This hints at a requirement of complete processivity of 18-hydroxylation and oxidation for A formation at naturally occurring concentrations. However, the mechanisms of intermediate turnover need further investigations comprising a wider range of concentrations as the binding affinity of intermediates is significantly reduced during the reaction sequence. Processivity of A formation in bovine is still a point of discussion as bovine CYP11B1 is able to oxidize free 18OH-B [109], although the main route of A formation is realized from DOC or B as a substrate, whereby A is formed successively [157]. Whether this need for processivity is substrate related or a general aspect of the 18-oxidase function can be investigated by studying the metabolism of F in more detail as F represents the second substrate that has so far been described for CYP11B2-dependent 18-hydroxylase and -oxidase activity [154,158]. The structural basis for the differentiated processivity and selectivity of both human isoforms was discussed in Ref. [127]. The authors compared the results of several previous studies, performed with the intention to identify function-specific residues in CYP11B enzymes (for review see Ref. [133]) with data of the human CYP11B2 structure and came to the conclusion that functional differences in gluco- and mineralocorticoid synthesis are mainly due to enhanced protein dynamics in CYP11B2 compared with CYP11B1 modulating the binding of substrates and the dissociation rates of hydroxylated intermediates.

In addition to their role in gluco- and mineralocorticoid synthesis, both human CYP11B enzymes have recently been proofed to be involved in transformations of progesterone and the 2 androgenic steroids, testosterone and androstenedione [127,159]. Their substrate spectrum, which has previously been regarded as rather narrow compared to e.g., liver P450s, therefore includes C21 steroids with and without 17- and 21-hydroxy groups as well as C19 steroids. The only shared structural element is the 3-keto-Δ4 motif of the A-ring and no metabolism of 3OH-Δ5 steroids has been observed so far. This restriction of structural motifs seems physiologically necessary, because it prevents direct metabolism of CYP11A1 products in the mitochondria. Hence, human CYP11B enzymes show additional, diverse functions in biotransformations of steroid hormones and a role in the derivatization of androgens, gestagens and F is indicated. Their 19-hydroxylase activity suggests a contribution to the biosynthesis of 19-normineralocorticoids by delivering 19-hydroxylated precursors. The finding that both CYP11B enzymes are also able to metabolize metandienone (17β-hydroxy-17α-methylandrosta-1,4-dien-3-on; 1,2-dehydromethyltestosterone) [160,161], a synthetic steroid that is frequently abused by professional and amateur sportsmen for doping purposes because of its anabolic effects, raises the question whether human CYP11B enzymes and adrenocortical P450s, in general, are involved in biotransformations of exogenous steroid-like compounds. The contribution of CYP11B1 in the biotransformation of methylsulfonyl-DDE (3-methylsulfonyl-2,2-bis-(4-chlorophenyl)-1,1-dichlorethen), a metabolite of the insecticide DDT (1,1,1-trichlor-2,2-bis-(4-chlorophenyl)ethan; dichlordiphenyltrichlorethan), in mice [162] even expands this aspect to nonsteroid-like molecules.

Taken together, the presented examples for CYP11B enzymes from different organisms reveal that the biosynthesis of gluco- and mineralocorticoids can be successfully performed by functional promiscuity of an individual enzyme, like in cattle, as well as by the evolvement of clear functional diversity between multiple isozymes of one organism, as presented for rat and human. The elucidation of the substrate space for the different enzymes supports the idea that there is no strict relationship between a steroid hydroxylase and its potential substrates as their interaction does not follow a simple lock-key principal.

3.2. Protein sequence variation and population genetics of human CYP11B enzymes

Already with the first publications about successful cloning and characterization of human CYP11B isozymes by different laboratories in the late 1980s and early 1990s [155,156,163], differences between the reported sequences on DNA and protein level could be discerned. Ever since the number of known sequence variants greatly increased, as major progress in the fields of DNA sequencing technologies and subsequent bioinformatical evaluation could be achieved, which enables today's systematic high-throughput analysis of human genes. Human CYP11B1 and CYP11B2 have been the target of such approaches in numerous studies as their essential role for the regulation of human metabolic and electrolyte homeostasis and immune response possibly results, in case of altered function and/or activity, in causal effects for common, especially cardiovascular, diseases. Thus, to understand the relationship between their genomic variation and disease can be a key in diagnosis, treatment and discovery of predisposition. The following section will briefly summarize the current state of our knowledge about sequence variation of these genes. Within that context we will focus on exonic, non-synonymous polymorphisms, in order to elucidate structural diversity on the protein level. Polymorphisms are defined as natural variants, that occur with an allele frequency of >1%. Individual, random mutations which are directly associated with disorders in steroidogenesis will not be included, as the purpose of this article is to highlight natural diversity and promiscuity of functional 11B enzymes.

Among human genes in general as well as in the group of genes encoding human steroidogenic cytochromes P450, the 2CYP11B genes belong to those with elevated genetic diversity. They display a variation frequency with a mean of about 1 single nucleotide polymorphism (SNP) per 100 base pairs in coding and non-coding regions, which represents about the 3-fold compared to the average SNP frequency of other genes involved in common diseases and regulation of blood pressure [164,165]. CYP21A2 is the only P450 involved in steroid biosynthesis, that shows a higher frequency of genetic variation (approximately 1 SNP per 80 bp) than CYP11B1 and CYP11B2 [164].

Table 1A and B summarizes the most frequent coding, non-synonymous polymorphisms occurring for human CYP11B1 and CYP11B2 and provide additional information about their localization in the protein molecule as well as results from *in vitro* activity studies [105,127,166]. The indicated allele frequencies are derived from the online browser of the 1000 genomes project (http://browser.1000genomes.org), the currently most stringent project for the identification of genetic variants in populations from

Table 1
Summary of the most common polymorphisms of human CYP11B1 (A) and CYP11B2 (B) including their respective average frequency in global population (1000 genomes browser), localization in the protein structure, indication of a potential origin in a gene conversion event with the other CYP11B isoform, and activity alterations in *in vitro* systems.

A – CYP11B1

Polymorphism	Average frequency (%)	Localization	Gene conversion	Effect on activity *in vitro*	Test system
R43Q	14	A-helix, outer surface	Yes	11β-Hydroxylation decreased to 50–30% with stronger effect on DOC conversion	Recomb. JEG-3 cells [181], substrates DOC and S
A386V	13	β1-4, outer surface	Yes	n.d.	–

B – CYP11B2

Polymorphism	Average frequency (%)	Localization	Gene conversion	Effect on activity *in vitro*			Test system
				11β-Hydroxylation	18-Hydroxylation	18-Oxidation	
K173R	36	D-helix, outer surface	No	Unaltered			Recomb. COS-7 cells [172], substrate DOC
I339T	12	J-helix, outer surface	No	Unaltered for DOC and S	Unaltered for DOC		Recomb. COS-1 cells [182], substrates DOC and S*
V386A	4	β1-4, outer surface	Yes	Unaltered	Decreased	Unaltered	Recomb. COS cells [179], substrate DOC
				Efficiency generally decreased			Recomb. COS-7 cells [172], substrate DOC
				Increased	Decreased	Unaltered	Recomb. *fission yeast* [177], substrate DOC
G435S	15	Loop between K′- and L-helix, outer surface	No	Unaltered for DOC	Decreased for B unaltered for DOC	Abolished	Purified protein from recomb. COS-7 [178], substrates DOC and B
				Unaltered	Decreased	Unaltered	Recomb. *fission yeast* [177], substrate DOC

* Analyzed for a CYP11B1/2 hybrid enzyme carrying the I339T exchange in comparison to CYP11B2 wt.

origins distributed all over the world, and represent the average frequencies in all individuals studied worldwide. However, for several polymorphisms characteristic allele distributions over populations with different ancestry can be observed, which nicely demonstrates, how classical evolutionary mechanisms, like genetic drifts and founder effects, reinforce biological diversity. While CYP11B1 R43Q and CYP11B2 G435S appear with an increased frequency of 43 and 48%, respectively, in populations from East Asian ancestry, CYP11B1 A386V and CYP11B2 I339T seem to be primarily restricted to populations with African ancestry, showing an elevated frequency of 38 and 44%, respectively, in these populations (for comparison with the corresponding mean values of frequency worldwide, see Table 1). The most common polymorphism in human CYP11B isozymes is the exchange of lysine in position 173 of CYP11B2 to arginine (K173R). In European populations, both alleles are almost equally distributed (K 52%, R 48%), but lysine is significantly more frequent in populations of African ancestry (81%). Iberian populations in Spain are the only European subpopulation exposing an increased frequency of 79% for the lysin allele, which has also been reported to occur predominantly in black individuals outside of Africa [167]. This might reflect African origin. Although the role of this polymorphism in primary aldosteronism and the development of hypertension has been intensively discussed, it turned out that the attribution of the K allele with hypertension results from a linkage disequilibrium between K173R and the -334C/T polymorphism in the promoter region, which alters gene expression and, in consequence, A level [168–171]. The amino acid exchange itself does not significantly affect CYP11B2 activity [172]. In addition to these well-known common polymorphisms, further benign variants of lower frequency can be discovered by screening small, unselected populations. Holloway et al. could identify 11 additional coding, non-synonymous polymorphisms of CYP11B2 with frequencies up to 4% by screening a number of 69 healthy, normotensive subjects. Eight of these (R87G, N281T, G288S, K296N, D335N, Q404R, A414P, H439Y) were investigated for B and A formation from DOC in a recombinant JEG-3 cell culture and all showed altered activity for at least one of the functions [173].

When considering the amino acid exchanges introduced by the quoted common polymorphisms it becomes obvious that the types of substitutions are very diverse with a range from conservative exchanges within the same class of amino acids (CYP11B1: A386V; CYP11B2: K173R and V386A) to more radical changes of the residue's chemical nature with the introduction of polar groups (CYP11B2: I339T and G435S) or the removal of a charge (CYP11B1: R43Q), which would be expected to threaten structural integrity of the enzyme. Despite alterations in catalytic activities for several polymorphisms (e.g., CYP11B1: R43Q; CYP11B2: V386A, G435S), that were observed by *in vitro* activity studies, these variants appear in healthwise unobtrusive subjects [172–176]. This leads to the hypothesis that human physiology tolerates CYP11B enzyme diversity concerning amino acid sequence up to a level, which might even lead to functional variations *in vivo*. Interestingly, individual amino acid exchanges seem to modulate the different reactions carried out by the CYP11B isozymes in distinct ways. For example, the V386A variant of CYP11B2, when expressed in fission yeast, exposes an increased activity for 11β-hydroxylation but a reduced activity for the respective reaction towards position 18, while the 18-oxidation activity remains unaltered [177]. Differentiated studies of enzyme variants *in vitro* can thus be an essential help to understand structure/function relation. However, limitations of *in vitro* systems in mimicking an authentic environment to a recombinant protein lead to disagreements between the results from different models, as it is the case for the data published about the effect of the V386A and G435R polymorphisms on CYP11B2 activity [172,177–179]. Still though, all studies support the thesis, that both variants especially reduce the efficiency of 18-hydroxylation.

When it comes to classifying the severity of the observed CYP11B diversity on protein level, 2 additional facts need to be taken into account. Firstly, the vast majority of polymorphisms concerns residues that are located on the protein surface

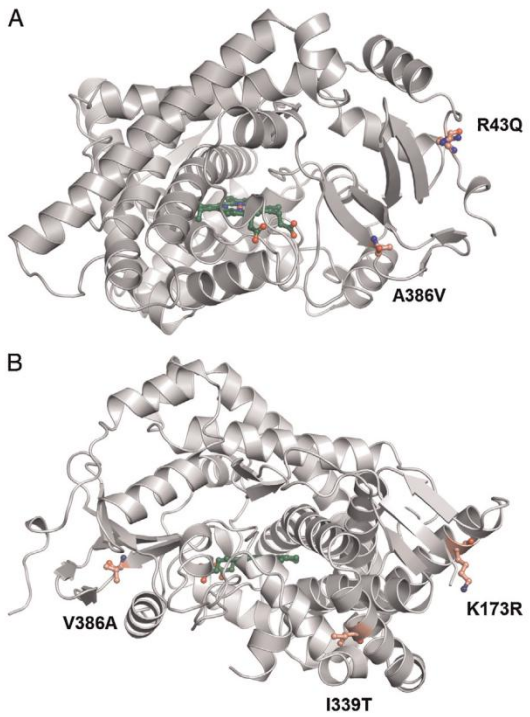

Fig. 4. Mapping of polymorphic residues in structure models of human CYP11B1 (A) and CYP11B2 (B). For the depiction of the CYP11B1 and CYP11B2 molecule the latest homology model [105] and the crystal structure [127] were used, respectively. For CYP11B2, G435S could not be mapped because of a gap in the structure.

(see Table 1 and CYP11B2: D335N, Q404R, A414P, H439Y described by Holloway et al. [173]) and consecutively do not have direct effects on essential events of the catalytic cycle in the active site (Fig. 4). However, changes in the flexibility of individual protein domains may affect the hydroxylation activity or selectivity. Secondly, a big number of polymorphisms (CYP11B1: R43Q, and A386V; CYP11B2: V386A and 9 out of 11 described by Refs. [173,180]) represent a substitution with an amino acid, that is present in the respective position of the other, highly homologous CYP11B isozyme, and presumably results from conversion events between the 2 genes. This might contribute to the maintenance of functionality despite the exchange, as long as key residues, which distinguish gluco- from mineralocorticoid synthesizing functions, are not involved. Therefore, natural diversity of human CYP11B isozymes on protein level can be ranged in as rather frequent, but generally leads to an only moderate degree of structural variation, which ensures functional integrity of the enzyme.

4. Conclusions

The diversity of CYP11B enzymes is omnipresent on multiple levels ranging from structural diversity of genes and proteins via specific transcriptional, translational and post-translational regulation mechanisms up to functional diversity and catalytic capacities. However, the underlying structural features and detailed regulatory mechanisms are still not sufficiently understood. Regarding the aspect of expression restriction to distinct zones of the adrenal cortex, which ensures a tissue specific production of gluco- and mineralocorticoids, modern techniques in epigenetics and the field of microRNA are promising novel approaches to get deeper insight into this challenging phenomenon. The expression of CYP11B isozymes in several organs other than the adrenals raises the question of a potential paracrine function in distinct extra-adrenal tissues. Zebra fish, whose role as model species in developmental studies is greatly increasing due to its genetic amenability and a highly conserved stress axis when compared to vertebrates, can serve as valuable tool with a completely revealed CYPome [183] for studies of stress-regulated steroidogenic mechanisms [184,185]. Although there are multiple indices for a determining role of post-translational modifications and especially phosphorylations for a modulation of the activity and product pattern of adrenal steroids, progress in this area is still limited and certainly needs further investigation. On the protein level, many open questions remain with regard to the isozyme specific catalytic properties. Structural characteristics and key residues that determine distinct functional specialization of different CYP11B enzymes and especially the capability to form A by an 18-oxidation, are not fully identified. Residues, which contribute to the differentiation of activity, could be narrowed down by mutagenesis studies [154,186,187] and the crystal structure of human CYP11B2 hints at a possible role of differences in conformational flexibility, but the detailed relations are not finally understood. In humans, all isozyme specific amino acids that have been studied and proofed to influence functionality are located between positions 284 and 339. However, in an alignment of this protein region with other CYP11B enzymes from different organisms expressing 1 or 2 isozymes (Fig. 5) no clear relationship between the encoded amino acids and functionality can be deduced. The only clear difference is glycine in position 288, which is present in all CYP11B2 structures as well as in the CYP11B proteins catalyzing F and A formation, but is absent in CYP11B1. This supports a hypothesis of a combinatorial effect of different

```
                                  Pos. 288        Pos. 301/302              Pos. 320          Pos. 335
Organisms with one enzyme
  Bos taurus                      WHYSGIVAELLMRADMTLDTIKANTIDLTAGSVDTLS-PLLMTLFELARNPEVQQAVRQE 342
  Ovies aries                     WHYSGIVAELLMRADMTLDTIKANTIDLTAGSVDTTAFPLLMTLFELARNPEVQQALRQE 343
  Rana catesbaiana                RGYSGIMAELLLQAELPLDSIKANITELMAGGVDTTAMPLLFTLFELARNPSVQRELREE 360
Organisms with two enzymes
  CYP11B1
  Homo sapiens                    QQYTSIVAELLLNAELSPDAIKANSMELTAGSVDTTVFPLLMTLFELARNPNVQQALRQE 343
  Mus musculus                    QSWS-VTAELVAERTLSMDAIQANSMELIAGSTDTTSTPLVMTFFELARNPDVQQALRQE 344
  Cavia porcellus                 QYNS-IVANLMLQGNLPLRAMKANIMDLVAGSVDTTALPLMMTLFELARNPTVQQALRQE 343
  CYP11B2
  Homo sapiens                    QHYTGIVAELLLKAELSLEAIKANSMELTAGSVDTTAFPLLMTLFELARNPDVQQILRQE 343
  Mus musculus                    QTYSGIVAELISQGSLPLDAIKANSMELTAGSVDTTAIPLVMTLFELARNPDVQKALRQE 345
  Cavia porcellus                 QYYSGIMADLLLQGDLSVNAIKANSIELTAGSVDTTAFPLMMTLFELARNSTMQQALHQE 344
                                     : : *:*: .  :.  :::**  :* **..**   **::*:******. :*: :::*
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Fig. 5. Alignment of CYP11B isoforms of 6 organisms (amino acid residues 284–342) containing either one or 2 enzymes. Residues identified as relevant for isozyme specific activity by mutagenesis studies with the human enzymes (*Homo sapiens*) are marked in red and were compared with the sequences of cattle (*Bos taurus*), sheep (*Ovies aries*), bullfrog (*Rana catesbaiana*), mouse (*Mus musculus*) and guinea pig (*Cavia porcellus*). Alignment was performed with the ClustalW2 multiple sequence alignment tool of the EMBL-EBI website.

positions on the 18-oxidation ability. Further investigations following structural alignments of functionally diverse CYP11B isozymes are hampered by the absence of 3D structures other than human CYP11B2. The structural resolution of a CYP11B1 isozyme without A forming activity as well as the resolution of a bifunctional isozyme, like the bovine one, able to produce F as well as A could raise new hints.

Additionally, the detailed reaction mechanisms and dynamics of A synthesis still need to be further elucidated in order to understand the integration of intermediate release and the need for processivity. Nevertheless, as human CYP11B1 and CYP11B2 are available in a purified form in larger amounts only since 2008 and 2012 [105,106], respectively, our understanding of the enzymatic function is still developing and certainly will lead to new insights into this demanding and important group of enzymes within the next few years.

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3. Discussion

3.1 Biotechnological oxyfunctionalization of glucocorticoids

Natural and synthetic glucocorticoids exhibit anti-inflammatory and immunosuppressive effects and represent the leading drugs in the treatment of allergies, inflammations and autoimmune diseases like multiple sclerosis. Originating from cortisol, new modified molecules were designed in the last decades, which exhibit a higher anti-inflammatory activity and a lower affinity to the mineralocorticoid receptor to reduce side effects. All of these pharmaceutical steroids have in common that they have to be hydroxylated at carbon atoms 11, 17 and 21, since these hydroxyl groups are essential for an interaction with the glucocorticoid receptor [57]. Chemically these hydroxylations at the steran skeleton are hard to perform and, thus, one is searching for a sustainable biocatalytic solution. The present work demonstrates the successful application of steroidogenic cytochromes P450, which are responsible for the biosynthesis of natural corticoids, for the refinement of steroids by highly selective hydroxylations. Human CYP11B1 as well as bovine CYP21A2 and their respective redox partners could be successfully expressed in an *Escherichia coli*-based whole-cell system for the selective production of the glucocorticoids cortisol and premedrol. Chemically performed hydroxylations, in general, consist of multiple steps producing many by-products, which have to be separated in time-consuming procedures. For this reason, protection groups are necessary, which afterwards have to be removed. Moreover, these reactions are catalyzed by iodine, which makes the procedure highly toxic and environmentally unfriendly [62-64]. The energetic expense further increases due to the need of harsh reaction conditions, like significant temperature differences during the reaction. The overall yield is in many cases not more than 30%, which is in terms of economics not profitable. The here demonstrated whole-cell systems offer a solution for a simple one-step hydroxylation, whereby toxic compounds, complicated purification steps due to unwished by-products and harsh conditions are swept away. Despite of the successful establishment of a sustainable biocatalytic process, these cytochrome P450-based systems still exhibit severe limitations, which have to be overcome for an industrial application [36, 37]. With regard to the reaction process, it is remarkable that the reaction stops several hours after initiation. In case of the CYP21A2-based reaction with arh1 and $etp1^{fd}$ as redox partners, the reaction already stops after 4 h in shaking flasks. The maximum yield of 320 $mg*L^{-1}$ is reached after 4 h, since the reaction is restricted to this

period, which might lead to the conclusion that 1.92 $g*L^{-1}$ could be achieved, if the reaction exhibited a linear course within 24 h. Therefore, it is of great importance to overcome restrictions, which impede an industrial application and make the biocatalytic approach less competitive, by maintaining the initial reaction velocity.

The work done on the CYP21A2-based whole-cell system showed the importance of a sufficient ***electron transfer*** by robust electron transfer proteins, which are easy to express and exhibit an optimal interaction with the CYP, since the system´s efficiency significantly increased with the replacement of the CPR by more soluble proteins, especially arh1 and $etp1^{fd}$ [74, 75]. To ensure an ideal interaction, an optimal ratio of the CYP to its redox partners is imperative. Although the reductase arh1 and the ferredoxin $etp1^{fd}$ were in excess over CYP21A2 as shown by Western blot analysis, a complete saturation of redox transfer proteins to exclude limiting effects was not achieved. Thus, it has to be considered that a higher expression could lead to an optimal stoichiometry leading to an improved conversion. One approach was followed when studying the CYP11B1 system by cloning multiple copies of the *Adx* gene into the polycistronic whole-cell system vector, consequently enhancing the biotransformation. Applied on the CYP21-system containing the Fpr and Adx as redox partners, two *Adx* cDNA copies only could improve the premedrol production by about 10% compared with about 40% in the CYP11B1-based system (Chapter 2.3) (Figure 7). Thereby, one has to take into account that Adx is not the natural redox partner of CYP21A2 and that these experiments clearly are worth to be repeated with $etp1^{fd}$ and arh1. Another solution is the use of distinct vector systems for the expression of the CYP and the redox partners with differently strong promoters. Janocha et al. showed even a tenfold excess of redox partners over CYP105A1 from *Streptomyces griseolus* using two distinct vector systems [76]. The vector pETDuet™-1 represents another alternative, as it carries two multiple cloning sites, each under the control of separated promoters, which allows to express genes with no or minimized polycistronic sequences.

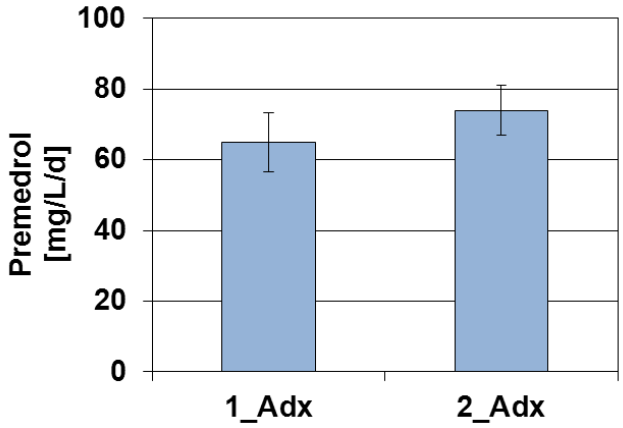

Figure 7: CYP21A2-based whole-cell medrane conversion with one or two copies of *Adx* cDNA. Premedrol amount gained from the whole-cell biotransformation with one or two copies of *Adx* cDNA arranged in a polycistronic manner with Fpr as reductase and with bovine CYP21A2.

An effective electron transfer is not only essential in terms of an efficient biotransformation, but also to minimize an electron leakage during the transfer. An uncoupling of electrons subsequently leads to the formation of ***reactive oxygen species*** (ROS) during the catalytic cycle of cytochromes P450 [48] (Figure 2). ROS are known to damage the prosthetic heme group irreversibly. Thus, ROS formation due to an electron uncoupling during biotransformation could explain why the reactions stops. So far, ROS formation has never been correlated experimentally to an uncoupling in a whole-cell system, neither was the ROS accumulation monitored during biotransformation. However, the impact of ROS on the biotransformation process needs to be elucidated in detail. One approach to cope with the damaging effects is to mimic nature, which compensates ROS formation enzymatically by proteins like the superoxide dismutase or catalase, which are able to transform radical substances to non-damaging water and oxygen [77, 78]. These proteins could be co-expressed to equalize ROS production effects and to prolong the system´s catalytic ability.

Although *E. coli* represents a suitable host for heterologous expression of CYPs, the whole-cell biotransformation might be hampered by an ***insufficient substrate uptake***. *E. coli* belongs to the gram-negative bacteria, which possess an outer membrane additionally to the cell wall and an inner membrane. Due to the special cell barrier composition, the diffusion of hydrophobic substrates like steroids into the cell is hindered and no special

transporter system is known to date. During the establishment of the whole-cell system this problem was overcome by testing several substances in a microtiter scale, which are known to permeabilize *E. coli´s* cell membrane to enhance the substrate access [76, 79].

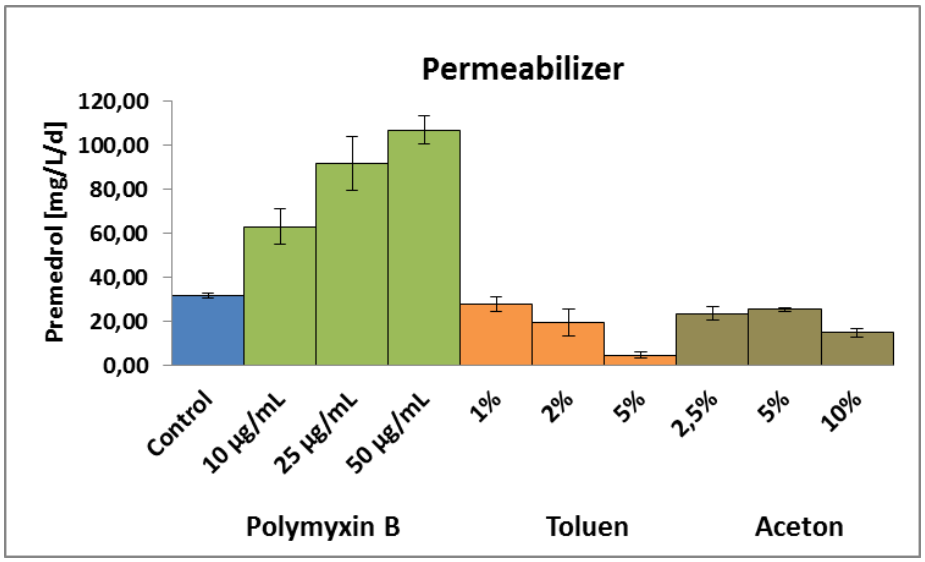

Figure 8: **Analysis of several *E. coli* permeabilizing agents.** Whole-cell biotransformation of medrane was carried out using different cell permeabilizing agents, which are investigated in view of the reaction enhancement.

Figure 8 shows that common solvents like toluene and acetone have negative effects on the product yield, while the antibiotic polymyxin B enhances the premedrol yield about 3-4 fold compared to the control without permeabilizer. Applied in a shaking flask scale with resting cells in buffer, polymyxin B led to a 2-fold improvement of the biocatalysis. Although the biotransformation could be improved, this permeabilizing antibiotic is not suitable for an industrial process in a large scale, since the costs would significantly increase and make the biocatalytic process less competitive. Thus, cheaper permeabilizing substances like EDTA or detergents like Triton X-100 have to be tested in the future [76, 80].

Beside the substrate access into the cell, the ***substrate solubility*** represents another severe bottleneck in CYP-mediated steroid biotransformation [81]. Due to the hydrophobic properties of steroids they are not miscible with the aqueous culture environment and precipitate and have to be diluted in solvents. Although ethanol is a suitable solvent for

steroids, it is not effective enough to produce highly concentrated stock solutions. Furthermore, *E. coli* is highly sensible towards ethanol and only tolerates concentrations up to 5%, whereby the application of higher substrate amounts is hard to handle without inducing toxic ethanol effects [82, 83]. In this study, DMSO was tested as an alternative to ethanol, and it was figured out that not only the substrate solubility was enhanced but also the corresponding premedrol formation. *E. coli* is able to convert DMSO by the enzyme dimethylsulfoxide reductase, a rare molybdenum-containing protein, to dimethylsulfide and is, for this reason, highly tolerant towards it [84, 85]. The ability of the DMSO reductase to transfer electrons to DMSO could make this solvent serving as scavenger molecule for ROS, which could explain the drastically increased product formation observed within CYP11B1- dependent biotransformation, which cannot only be explained by a higher solubility (chapter 2.3). Therefore, DMSO is a suitable solvent and co-solvent for steroids and ensures an appropriate amount of accessible substrate circulating in the system. In context with substrate access, a further challenge represents the establishment of an appropriate ***in-situ product removal (ISPR)*** technique, since the hydroxylated product premedrol is much more water-soluble than the substrate medrane, which subsequently leads to an accumulation of product in the aqueous phase resulting in a possible product inhibition [86, 87]. Titration of bovine CYP21A2 with premedrol results in a type I shift with an estimated K_D value of 116 ± 10 µM, which is a clear indication for product binding and emphasizes the need of a product removal in future applications. One approach is the exploitation of the different hydrophobic properties of substrate and product, which results in a different affinity to solvents that could be used in a two-phase system [88, 89]. In recent years, resins like Amberlite™ have become of growing interest, since they represent a suitable tool to separate molecules from solutions through their hydrophobicity or polarity [90]. Though they are simple to handle, it takes much time and effort to find a resin, which selectively binds the product.

In this work, a whole-cell system for the production of premedrol was chosen in preference of an *in vitro* approach with purified proteins, since the proteins remain stable inside the cell and do not need to be isolated via long-lasting purification procedures. Furthermore, the costly cofactor NADPH is provided by the host and does not need to be supplemented, whereby our chosen host *E. coli* in combination with glycerol seems to be the best choice regarding the cofactor regeneration ability according to literature data [91]. Despite of this

obvious advantage, the ***cofactor accessibility*** represents a crucial bottleneck regarding an industrial process, which could lead to a stagnation of the hydroxylation reaction, as the host´s natural NADPH capacity is limited. To overcome NADPH limitation, the whole-cell biotransformation was carried out in buffer medium, which leads to a transition of the cells into a resting stage after protein expression. In this state, the cell´s metabolism as well as protein biosynthesis is restricted to a minimum, since the buffer lacks nitrogen sources and amino acids [92-94]. For this reason, reducing equivalents like NADPH, which mainly are involved in anabolic processes, can be recruited for the CYP-mediated hydroxylation reaction. Furthermore, the addition of a carbohydrate source to the reaction buffer serves for NADPH regeneration from $NADP^+$ through the pentose phosphate pathway by the glucose-6-phosphate dehydrogenase and through the citrate cycle involved enzyme isocitrate dehydrogenase [95]. While biotransformation in complex medium was reduced to a minimum during initial investigations of the established system, the reaction velocity and total product yield increased significantly by using resting *E. coli* cells, which might be due to more accessible NADPH. Within mammalian electron transfer systems, the reductases CPR and AdR are only able to abstract electrons from NADPH, which is less abundant in *E. coli* than the non-phosphorylated NADH during a normal metabolic state [96, 97]. In this work, we managed to tap another electron source by using the *S. pombe* reductase arh1, which is able to abstract electrons from NADPH as well as from NADH for biotransformation, as shown in *in vitro* assays with purified enzymes (chapter 2.1). Dependent on the reductase, the reaction velocity as well as the overall premedrol yield could significantly be improved by the use of arh1 in the CYP21A2-based whole-cell system, emphasizing the importance of a sufficient pool of reducing equivalents. Aside from the NADPH-regeneration by supplemented carbohydrates, the targeted coexpression of NADPH-regenerating enzymes like the glucose-6-phosphate dehydrogenase, phosphite dehydrogenase or the lactic acid dehydrogenase was performed in other studies [76, 98, 99]. With regard of a stable, long-lasting biotransformation this kind of cofactor regeneration has to be considered in the future for a competitive biocatalytic premedrol production. To exclude a fast depletion of the buffer medium and the carbohydrate source, the cells were transferred into new buffer after 2 hours and 4 hours with the result, that no significant difference between biotransformation with cells in fresh medium and cells persisting in the initial reaction buffer could be observed (Figure 9).

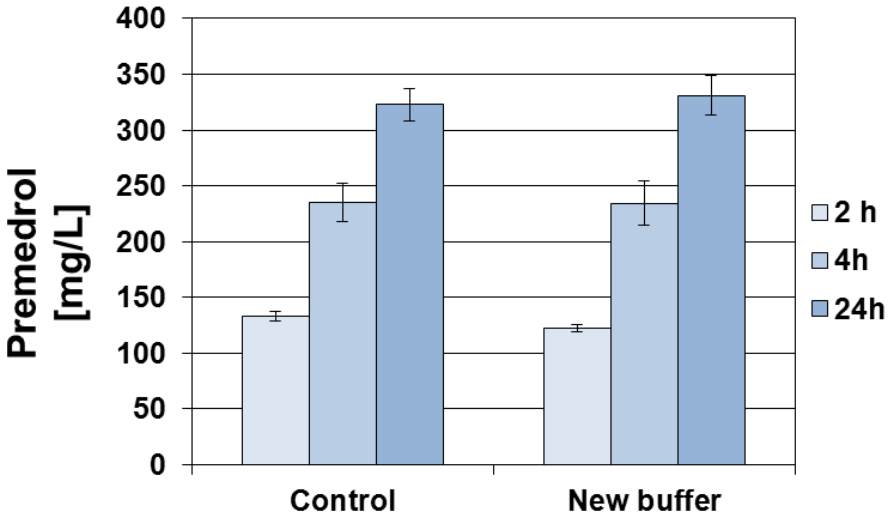

Figure 9: **Impact of buffer refreshment on the biotransformation rate.** The reaction buffer was renewed after 2 and 4 hours biotransformation to examine an exhaustion of carbohydrate source and to enhance biotransformation of medrane by the CYP21A2-dependent whole-cell system.

Taken together, the reasons why CYP-mediated whole-cell biotransformations stop after several hours have not been completely identified yet and have to be elucidated in detail in the future to be eliminate them for the establishment of an effective biotechnological process.

3.2 CYP21A2 as biocatalyst

In general, cytochromes P450 exhibit low turnover numbers and, therefore, have to be improved for a profitable industrial application. In this work, two isoforms of CYP21A2 were tested regarding their biocatalytic ability. Starting with bovine CYP21A2, whose expression has been already successfully established in *E. coli*, the CYP21A2 amino acid sequence was aligned with CYP21 sequences of other species. It was figured out, that the human CYP21A2 exhibits a sequence homology of 79% and, therefore, was chosen to test as a second CYP21 isoform in a whole-cell system. Similarly to bovine CYP21A2, the human CYP21A2 gene sequence was modified by an N-terminal truncation to remove

hydrophobic anchor regions enhancing a soluble expression, since former expression procedures only achieved poor yields [70, 100]. With the adapted gene sequence, the expression level for human CYP21A2 increased up to 327 nmol/L culture, which corresponds well with 398 nmol/L culture for the bovine isoform. Both isoforms were able to convert medrane to premedrol in *in vitro* assays, so that vectors for whole-cell biocatalysis were constructed for both enzymes. Comparative biotransformation resulted in a significantly higher product formation in case of the bovine CYP21A2 with the four tested redox systems (Figure 10). Kinetic studies in context with the crystal structure for human CYP21A2 revealed in 2015 that the human enzyme exhibits a much higher activity than the bovine one in case of the natural substrates progesterone and 17OH-progesterone, which is at first glance, however, contradictory to the data obtained here with medrane *in vivo* [101].

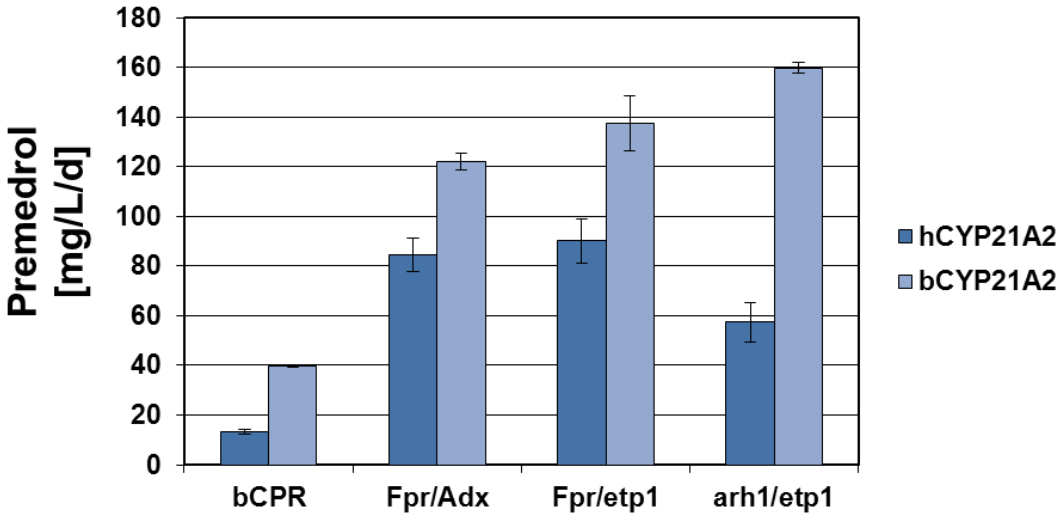

Figure 10: Comparison of the whole-cell systems based on bovine and human CYP21A2. Bovine and human CYP21A2-based whole-cell systems were compared with four different redox systems in view of their time-space-yield of premedrol.

One has, however, to take into account that the data produced by Pallan et al. was achieved with purified enzymes and another isoform of the CPR, the rat instead of the bovine one, which could lead to an altered activity [101]. This study impressively shows the dependence of CYP activity on the choice of redox partners. Moreover, whole-cell

catalysis is dependent from more than one factor, not as controllable as *in vitro* assays and often leads to other results compared with purified enzymes. It was shown that the bovine isoform is highly stable, even after 24 h biotransformation. The stability of the human enzyme should be verified in the future by COD spectroscopy to exclude a stability effect. Additionally, the synthetic glucocorticoid medrane used as a substrate here is not a natural substrate of CYP21A2 and differs from 17OH-progesterone by an additional methyl group at C6 and an 11β-hydroxylation. Despite of the high sequence similarity, the crystal structures of the human CYP21A2 isoform show some crucial differences to the bovine one elucidated in 2012 [102]. Although the amino acids of the active side are highly conserved, there are some changes with a possible impact:

- **Gly-468** (bovine) instead of **Val-470** (human): Gly-468 is supposed to be responsible for a second distal substrate binding pocket in the bovine CYP21A2. The impact of a second binding cavity is not clear yet, but seems to facilitate the substrate binding in the active site. An exchange by a larger amino acid could disrupt an interaction.
- **Ser-97** (bovine) instead of **Thr-97** (human): Position 97 is near to the residues responsible for closing the active site. It is not known if this change has an impact.
- **Met-197** (bovine) instead of **Leu-199** (human): In case of a medrane conversion, this change seems to be the most crucial one, since this residue interacts with the C11 of the steroid substrate. In contrast to the natural substrates, the C11 of medrane is hydroxylated. Since methionine is two times less hydrophobic than leucine according the Kyte and Doolittle scale, it is more suitable to stabilize the hydroxyl group than the hydrophobic leucine. To verify this hypothesis, the dissociation constant for medrane titrated to the human enzyme should be determined in the future and compared to the K_D value for the bovine isoform.

For a future improvement of the CY21A2 application for premedrol production, techniques of enzyme engineering by molecular evolution should be applied to increase the catalytic activity as well as the binding to the substrate medrane, whose K_D value is about 100 times higher than that for the natural substrates. For this, the higher catalytic efficiency of the human enzyme should be combined with the higher output in a whole-cell system using the bovine one. For this, an exchange of Leu-199 to Met-199 in the human CYP21A2 by site-directed mutagenesis would be very exciting to examine the C11-interacting position, possibly leading to a better medrane binding, using the higher activity of the human

enzyme. Since it would be very time-consuming to examine all residues to elucidate the ones responsible for the higher activity and to introduce them one by one into the bovine isoform, a gene shuffling of bovine and human CYP21A2 represents a fast method to generate a mutant library with hybrid DNA sequences, which may exhibit a higher activity in whole cells [103]. In general, site-directed mutagenesis represents a powerful tool to improve an enzyme and to determine crucial residues, also resolving functional protein properties. The above-mentioned residue Met-197 could serve as target for saturation mutagenesis, finding amino acids, which further stabilize the hydroxyl-group at C11 and, therefore, improve the medrane binding. The molecular evolution done on the human CYP11B1 (Chapter 2.3) revealed residues improving the enzyme´s application in a whole-cell system regarding the initial velocity as well as its stable expression. Alignment of the bovine CYP21A2 sequence with the CYP11B1 sequence shows, which residues could have an impact on the CYP21A2-system. Since the CYP11B1_G23R mutant (shortened enzyme sequence) significantly increases the stability and the biotransformation rate and, moreover, is a conserved residue in bovine CYP21A2, the Gly-36 (wild-type enzyme sequence) was exchanged by an arginine via site-directed mutagenesis.

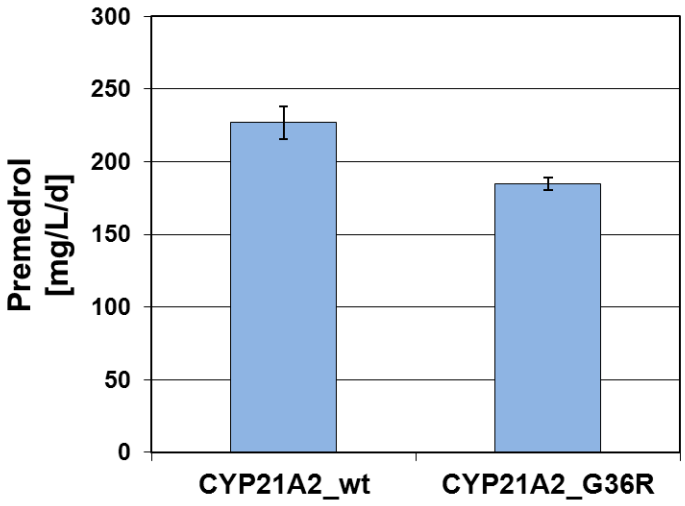

Figure 11: Comparative biotransformation of medrane by CYP21A2 and CYP21A2_G36R. Wild type CYP21A2 and the mutant CYP21A2_G36R were compared in biotransformations regarding their premedrol endpoint yield.

Product analysis revealed that the beneficial effect of this amino acid exchange in hCYP11B1 could not be transferred to bCYP21A2, as the total product yield decreased about 20% compared to the wild type sequence (Figure 11). The other described CYP11B1-activity increasing mutations might have no obvious impact on CYP21A2 activity, since the corresponding residues mainly contribute to the structural integrity of the protein scaffold. In general, mutations within the active site of CYP21A2 are critical, since it has a unique amino acid arrangement enabling the energetically unfavored hydroxylation at C21 by forming a relatively unstable carbon radical and a tight cavity avoiding energetically more favored hydroxylation reactions like 16α- and 17α-hydroxylations [104]. Thus, aside from a site-directed mutagenesis a random mutagenesis should additionally be aspired, to generate mutations with an impact on redox partner interactions, protein stability and increase of the catalytic ability. For this, a suitable screening system is imperative, selecting the desired property. This issue will be treated in the next section.

3.3 A screening system for CYP-mediated catalysis

According to the slogan "You get what you screen for", the development of a suitable screening system for mutants with an increased activity has to be intended for enhanced product formation. Lent on the already established system for hCYP11B1 (**Chapter 2.3**), a screening system for the molecular evolution of CYP21A2 was developed within the scope of this work, which uses the higher fluorescence of hydroxylated steroids in an acidic environment [105]. In initial examinations it was verified that premedrol exhibits a higher relative fluorescence than the substrate medrane, which was the basis for an application of a fluorescence-based screening assay. Since the screening system should be established in a microtiter scale, the expression of bovine CYP21A2 together with its most sufficient redox partners, arh1 and etp1^{fd}, and a functional medrane conversion had to be ensured without performing additional steps like the transfer into buffer to exclude disturbing medium effects. For this, the *tryptophanase* gene (*tnaA*) was successfully disrupted in the *E. coli* strain C43(DE3) resulting in the strain C43(DE3)_Δ*tnaA*, which is highly suitable for the expression of CYP systems. The tryptophanase is responsible for the synthesis of the aromatic compound indole, which is supposed to be a strong inhibitor of CYP-mediated biotransformation as shown in **chapter 2.2** and originates from the amino acid tryptophan [106-108]. Biotransformation with CYP21A2 was enhanced by about 30% by

using the C43(DE3)_Δ*tnaA* strain in a microtiter scale, while an enhanced protein expression rate was observed. After a successful medrane conversion was verified by HPLC analysis, the fluorescence-based assay was applied for whole cells. It was shown that the induced culture exhibited a higher fluorescence than the not induced one due to the abundancy of medrane-hydroxylating CYP21A2 so that this screening method is supposed to be convenient for an undirected mutagenesis (Figure 12). Therefore, an undirected mutagenesis via error-prone PCR was carried out and, so far, 100 mutants were screened by this fast three-days-lasting screening method. Taken together, the established screening system is highly suitable for site directed as well as undirected mutagenesis for the selection of mutants with improved hydroxylation activity.

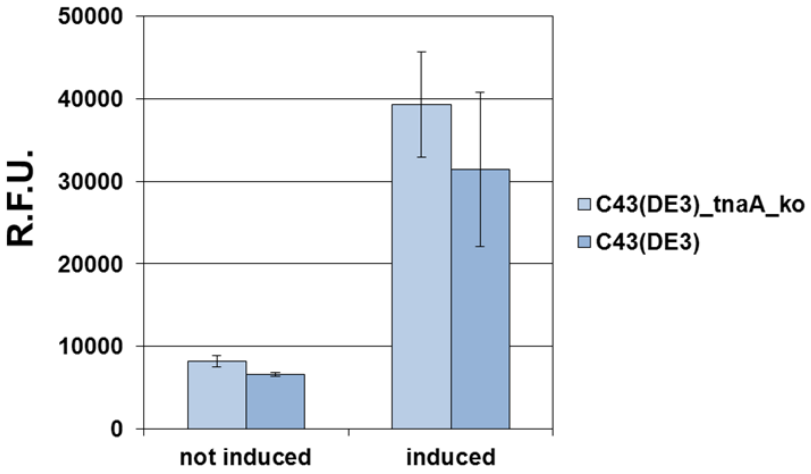

Figure 12: Application of a fluorescence-based assay on the CYP21A2 whole-cell system. CYP21A2 and the redox partners arh1 and etp1^{fd} were expressed in the strain C43(DE3) and the indole deficient strain in 1 mL. Biotransformation was performed with 300 µM medrane. The fluorescence assay was applied on induced and not induced cells showing higher relative fluorescence intensity (R.F.U.) in induced cultures due to medrane hydroxylation to premedrol.

In general, the screening of cytochromes P450 is getting growing interest in context of the molecular evolution of known CYPs for a wished property as well as the exploitation of orphan CYPs to elucidate their substrate spectrum and natural function. Since the number of orphan CYPs is significantly increasing, efforts have to be undertaken to facilitate and

to accelerate screening and exploitation. In **chapter 2.2** it was shown that the created *tnaA* knock out strain of C43(DE3), which is no longer capable to produce the CYP inhibitor indole, is highly suitable for the screening of CYPs of the myxobacteria *Sorangium cellulosum* So ce56, which can be considered as a model for the screening of orphan CYPs. Starting from the indole-deficient *E. coli* strain, the idea rose to create a CYP-adapted strain to facilitate expression and screening of orphan CYPs in a miniaturized scale. CYP expression based on inducible systems requires crucial additives like inducing agents (e.g. IPTG) and δ-aminolevulinic acid, which is a heme precursor needed for the biosynthesis of the prosthetic group. *E. coli* does not have an excess of circulating heme because of its toxic effects and for this reason the heme biosynthesis is strictly regulated resulting in a deficiency of free heme for CYP synthesis. In 2001 it was shown that the expression of various CYPs was enhanced without the use of δ-aminolevulinic acid by overexpression of the *hemA* gene encoding the enzyme glutamyl-tRNA reductase [109]. This enzyme catalyzes the committed step of the heme biosynthesis in *E. coli* and, hence, is highly regulated [110, 111]. Inspired by this work, initial effort was undertaken in the scope of this Thesis to integrate the *hemA* gene locus into the *E. coli* genome to avoid the addition of the heme precursor to the whole-cell system. For this, the *hemA* gene sequence was amplified out of the C43(DE3) genome and cloned into a pET17b-based vector together with human CYP21A2 in a bicistronic manner. For a proof of principle, the expression yield of human CYP21A2 co-expressed with *hemA* compared with a common expression with the addition of δ-Ala was examined. Spectral analysis resulted in an expression yield of 189 nmol/L culture for the control expression and a yield of 229 nmol/L culture for CYP21A2 co-expressed with *hemA*, indicating a successful replacement of δ-aminolevulinic acid. In view of a future application, it is planned to perform a gene knock-in of a controllable *hemA* containing operon into the genome of C43(DE3) for a stable, vector-independent expression without supplemented δ-Ala, which would reduce costs of approx. 8 € per liter [112].

3.4 Outlook

Within the scope of this Thesis it was shown that steroidogenic cytochromes P450 applied in biocatalytic whole-cell systems exhibit high potential for the production of pharmaceutically relevant glucocorticoids as well as steroid-originating metabolites.

Nevertheless, the established systems are restricted to only a few hours of activity and the reasons have to be evaluated in the future to diminish limiting factors. Although shaking flasks are convenient tools to develop processes on a basic level for initial investigations in a lab scale, an optimization has to be performed in a controllable system to minimize disturbing factors. For an enzymatic application it is imperative to maintain stable pH and temperature, which should be situated within the enzyme´s optimum. During *E. coli* cultivation in shaking flasks, the bacteria´s metabolism switches from an aerobe state to an anaerobe mixed acid fermentation due to an oxygen depletion, which leads to an accumulation of acetate [113]. Thus, the medium is acidified and the pH drops under a value of 6, which clearly impairs the catalytic activity of a mammalian CYP, whose pH optimum is in a physiological range between 7.2 and 7.4. Therefore, the transfer of the system from flasks to a controllable fermentation system, by which pH and temperature can be adjusted, is of great interest to maintain the high initial biocatalytic activity. During shaking flask cultivation, the oxygen supply is insufficient, though essential for a CYP-mediated reaction, and should be enhanced with the introduction of molecular oxygen additionally to an adapted stir velocity to maintain the reaction. One has to take into account that protein expression and biotransformation have to be considered as separated processes to be optimized in view of the development of an industrial biotransformation with CYP21A2 in a bioreactor. For example, while expression of CYPs does not necessarily require oxygen for a high yield, it is crucial for the following biotransformation. That means that every parameter has to be adjusted on the one hand for CYP expression and on the other hand for CYP-mediated biotransformation as shown in [114]. In view of an industrial application, the CYP21A2-based whole-cell system clearly represents a promising starting point producing 0.65 $g*L^{-1}$ in an uncontrollable Biostat® system and leaves room for improvement. Another future aspect will definitely be an enzyme engineering of CYP21A2 by molecular evolution consisting of site-directed and random mutagenesis. The main focus will be laid on an improved binding of the synthetic substrate medrane as well as on an improvement of the catalytic activity, whereby the residue Met-197 will surely be object of mutation approaches. In this context, the generated C43(DE3)_Δ*tnaA* strain could be taken as starting point to create an *E. coli* strain adapted to CYP expression and biotransformation. For this, a genomic integration of the above-mentioned *hemA* gene locus could be taken into consideration. Aside from a

hemA gene integration, the genome-originating coexpression of other proteins essential for CYP expression and biotransformation could be taken in consideration. The *E. coli* chaperones GroEL/ES, which are needed for a correct protein folding, as well as redox partner proteins, which are known to interact with a broad spectrum of CYPs like arh1 and etp1^{fd} could be integrated into the genome leading to the avoidance of polycistronic gene arrangements and antibiotic-dependent vectors [115]. This fictive CYP-adapted *E. coli* strain could serve as powerful tool for simplified screening procedures, since only the introduction of the CYP-containing plasmid would be required without caring about chaperones, redox partners and further additives (Figure 13). The cultivation would be enhanced by using the same promoter for each expressed gene, which is inducible by using an autoinduction medium making IPTG redundant [116].

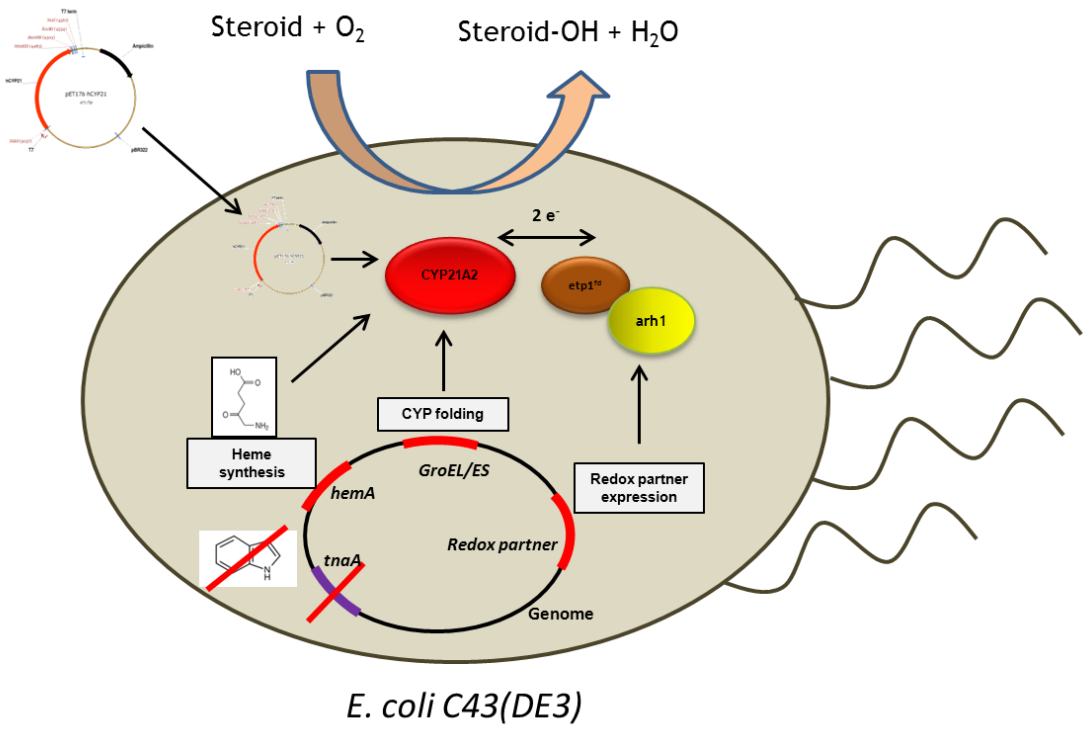

Figure 13: **Future CYP-adapted *E. coli* strain.** The schematically presented *E. coli* strain is genetically adapted for a facilitated CYP expression and CYP-based biotransformation. The strain is indole deficient due to a tryptophanase knock out. Furthermore, the genes for chaperones, redox proteins and *hemA* are integrated into the genome.

Taken together, in the scope of this Thesis it could be shown that mammalian CYPs are promising candidates for biocatalysis to make industrial processes more sustainable. Furthermore, fundamental work was done for a protein engineering of CYP21A2 as well as an elucidation of orphan CYPomes by establishing a screening system applying an indole deficient *E. coli* strain.

4. Abbreviations

Adx	Adrenodoxin
AdR	Adrenodoxin reductase
arh1	Adrenodoxin reductase homologue 1
CYP, P450	Cytochrome P450
CYP21A2	21-hydroxylase
DMSO	dimethyl sulfoxide
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
$etp1^{fd}$	Electron transfer protein 1
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
HPLC	High performance liquid chromatography
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
K_D	Dissociation constant
S. cellulosum So ce56	*Sorangium cellulosum* So ce56
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*

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