

Expression of human steroid hydroxylases in
fission yeast

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Contents

List of Figures	vi
List of Tables	vii
Abbreviations	viii
Symbols and variables	ix
Notes on nomenclature and style	x
Abstract	xi
Zusammenfassung	xii
Scientific contributions	xiii
1 Introduction	1
1.1 Steroids as chemical entities	1
1.2 Adrenal steroids	3
1.3 Clinical aspects of steroid biosynthesis	6
1.4 Steroid synthesis	9
1.4.1 Biocatalysis by P450s	9
1.4.2 Chemical synthesis	18
1.5 The rationale for this work	20
1.5.1 Focus on recombinant whole-cell biotrans- formation	20



1.5.2	Use of human P450s and fission yeast . . .	22
1.6	Aims of this work	27
2	Discussion	28
2.1	Functional expression of human CYP11B1 in fission yeast	28
2.1.1	The human CYP11B1 is expressed and correctly localized in fission yeast cells	29
2.1.2	Fission yeast strains expressing the human enzyme CYP11B1 convert 11-deoxycortisol to cortisol in vivo	30
2.1.3	Space-time yield on cortisol	32
2.1.4	Fission yeast electronically sustains mitochondrial P450 reactions	34
2.1.5	The human CYP11B1 and CYP11B2 show different kinetic properties when expressed in fission yeast	36
2.1.6	Application of CYP11B1 expressing fission yeast strains for inhibition studies	40
2.2	Functional expression of the microsomal human P450s CYP17A1 and CYP21A1 in fission yeast .	41
2.2.1	Expression of microsomal P450s	41
2.2.2	Human CYP17A1 and CYP21A1 are functionally expressed in fission yeast	42
2.2.3	Application of CYP17A1 and CYP21A1 expressing fission yeast strains for inhibition studies	45
2.2.4	Electronic coupling of microsomal P450s to host systems and its implications	47
	Conclusions	51
	List of research papers in chronological order	52



Acknowledgments	54
A Proteins, peptides, genes, and DNA sequences	55
B Dragan et al. (2005)	56
C Dragan et al. (2006a)	62
D Dragan et al. (2006b)	79
Bibliography	90



List of Figures

1.1	Chemical structure of lanosterol.	2
1.2	Ring designations and atom numbering in steroids	2
1.3	An exemplary conformer of cholesterol	3
1.4	Histology of the human adrenal	4
1.5	Steroid biosynthesis pathways in the human adrenal	5
1.6	Topology and structure of P450s	11
1.7	Hypothetical P450 reaction cycle	13
1.8	Starting structures for partial steroid syntheses .	19
2.1	Modification of a 1.5 mL tube to yield a tip-tube	41



List of Tables

1.1	Taxonomy of fission yeast	23
2.1	Fission yeast strains used in this work	29
A.1	Proteins, peptides, genes, and DNA sequences used in this work	55

Abbreviations

Abbreviation	Representation
16Prog	16 α -hydroxyprogesterone
17Prog	17 α -hydroxyprogesterone
18B	18 β -hydroxycorticosterone
aa	amino acid(s)
Aldo	aldosterone
B	corticosterone
bp	base pair
Bp	byproduct
CAH	congenital adrenal hyperplasia
cDNA	complementary DNA
CHO	Chinese hamster ovary, cell line
CMV	cytomegalovirus
COS	African green monkey CV-1 cell line carrying SV-40
CBR	cytochrome b ₅ reductase
CPR	cytochrome P450 reductase
CYP	cytochrome P450
cyt b ₅	cytochrome b ₅
DAD	diode array detector
DHEA	dehydroepiandrosterone
DISC	discontinuous
DNA	deoxyribonucleic acid
DOC	11-deoxycorticosterone
EMM	Edinburgh Minimal Medium
F	cortisol
Fdx	ferredoxin
GRAS	generally regarded as safe
HPLC	high pressure liquid chromatography
HSD	hydroxysteroid dehydrogenase
IUB	International Union of Biochemistry
IUPAC	International Union of Pure and Applied Chemistry
LLE	liquid-liquid extraction
NAD	nicotinamid adenine dinucleotide
NADP	nicotinamid adenine dinucleotide phosphate
NCYC	National Collection of Yeast Cultures
nt	nucleotide
P	product
PAGE	polyacrylamide gel electrophoresis
PPP	pentose phosphate pathway
Prog	progesterone
ROS	reactive oxygen species
S	substrate
SDS	sodium dodecyl sulfate
SREBP	sterol regulatory element binding protein
TLC	thin layer chromatography
V79	Chinese hamster lung fibroblast cell line

Symbols and variables

Abbreviation	Representation	Unit
c	concentration	M
$c(t)$	concentration at time t	M
$\langle CR \rangle$	average space-time yield	$\mu\text{M d}^{-1}$
g	earth's centripetal acceleration	m s^{-2}
IC_{50}	inhibitor concentration at which 50 % activity	μM
$K_{1/2}$	pseudo-Michaelis-Menten constant	μM
λ	wavelength	nm
t	time	d
v_{\max}	maximum conversion velocity	M s^{-1}

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Notes on nomenclature and style

All nomenclature uses accepted standard symbols as far as possible. Units are typeset according to SI. Element nomenclature follows IUPAC rules. Biochemical nomenclature follows IUB recommendations.

Carbon atoms belonging to a molecule are addressed as 'C- n ' where 'C' stands for carbon and n denotes the atom number according to IUPAC rules. The total number of carbon atoms of a molecule is abbreviated as 'C $_n$ ' with 'C' again standing for carbon and n being the carbon atom count.

A redox reaction of the form $X_{\text{red}} + Y_{\text{ox}} \rightarrow X_{\text{ox}} + Y_{\text{red}}$ is often additionally given in a simplified form as $X \rightarrow Y$ to emphasize the vectorial flow of electrons.

Enzyme names are often typeset as ' $_s$ Protein' with 's' denoting the source species where 's' can be substituted by either 'b' for bovine, 'h' for human, or 'r' for rat proteins. Fission yeast proteins are not labeled by a specific prefix subscript. Species names as well as gene names are typeset in italics according to general rules.

Abstract

Genetically modified microbial organisms are being increasingly used for the industrial production of complicated chemical compounds such as steroids; however, prior to this work there have been few reports on the use of the fission yeast *Schizosaccharomyces pombe* for this purpose. In the human adrenal, the mitochondrial P450 enzyme CYP11B1 catalyzes the conversion of 11-deoxycortisol to cortisol while the microsomal P450s CYP17A1 and CYP21A1 together with the aldo-keto reductase 3 β -HSD perform the crucial conversion of pregnenolone to either mineralocorticoid, glucocorticoid, and sex steroid precursors. Expression of these P450s in fission yeast resulted in strains that show a considerable biotransformation activity in whole-cell assays carried out over several days. The host is capable of supplying these enzymes with the reducing equivalents necessary for steroid hydroxylation activity indifferent of whether the P450s are localized in mitochondria or in the endoplasmic reticulum.

Zusammenfassung

Gentechnisch veränderte Organismen werden zunehmend für die industrielle Produktion komplizierter chemischer Verbindungen wie Steroide eingesetzt, jedoch gab es vor dieser Arbeit relativ wenige Studien über die Verwendung der Spalthefe *Schizosaccharomyces pombe* für diesen Zweck. In der menschlichen Nebenniere katalisiert das mitochondriale P450 Enzym CYP11B1 die Umwandlung von 11-Desoxycortisol zu Cortisol, während die mikrosomalen P450 Enzyme CYP17A1 und CYP21A1 zusammen mit der Aldo-Keto Reduktase 3 β -HSD die essentielle Umwandlung von Pregnenolon zu Mineralokortikoid-, Glukokortikoid- und Sexualsteroid-Vorstufen katalysieren. Die Expression der genannten P450 Enzyme in der Spalthefe führt zu Stämmen, welche eine beachtliche Ganzzell-Biotransformationsaktivität über mehrere Tagen zeigen. Der Wirtsorganismus ist dabei in der Lage, die für die Steroidhydroxylierung notwendigen Reduktionsäquivalente bereitzustellen, gleichgültig, ob es sich dabei um mitochondriale oder mikrosomale P450 handelt.



Scientific contributions

This work is based on the three research papers contained in the appendix. The original electronic copies are reproduced with kind permissions from John Wiley and Sons for Dragan et al. (2005) and Dragan et al. (2006a) and from Informa Healthcare Communications for Dragan et al. (2006b).

Dragan et al. (2005)

The author constructed the expression vector pCAD1 and developed the biotransformation assay. He contributed to the construction of the fission yeast strain SZ1, performed the biotransformation of 11-deoxycortisol, and analyzed the data. He was also involved in preliminary studies concerning the electrophoretic-Western detection of the expressed CYP11B1 and contributed to writing the manuscript.

Dragan et al. (2006a)

The author constructed the CYP21A1 expression plasmid and generated the fission yeast strain CAD18. He developed the following procedures: chromatographic methods for steroid analysis, oxygen consumption rate measurements, cytometric detection of reactive oxygen species, and reaction simulation. The following experiments were performed by the author: parts of the electrophoretic-Western detection of the expressed enzyme, the

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biotransformation assays, the oxygen consumption rate measurements, and detection of reactive oxygen species. Experimental data stemming from the mentioned experiments were analyzed by the author. The author programmed and performed the reaction simulation. He also contributed to writing the manuscript.

Dragan et al. (2006b)

The author constructed the CYP17A1 expression plasmid and generated the fission yeast strain CAD8. He developed the following procedures: chromatographic methods for steroid analysis, IC_{50} determination measurement, and reaction simulation. The following experiments were performed by the author: parts of the electrophoretic-Western detection of the expressed enzyme, the biotransformation assays, and IC_{50} measurement. Experimental data stemming from the mentioned experiments were analyzed by the author. The author programmed and performed the reaction simulation and contributed to writing the manuscript.

Chapter 1

Introduction

1.1 Steroids as chemical entities

Biosynthetically seen, steroids are the product of successive condensation reactions of the C₅-body isopentenyl pyrophosphate and its double bond isomer dimethylallyl pyrophosphate yielding C_{5n}-bodies. Squalene, precursor to cholesterol and, therefore, to all steroid structures, is synthesized by the condensation of two farnesyl pyrophosphate units which are C₁₅-bodies belonging to sesquiterpenes. Because squalene is a C₃₀-body, the biosynthetic class it is assigned to is called triterpenes. The oxidation of squalene by the squalene monooxygenase (*SQLE* in *Homo sapiens*, *erg1* in fission yeast) is followed by the generation of a carbocation through the action of lanosterol synthase (*LSS* in *Homo sapiens*, *erg7* in fission yeast) which immediately induces a group migration-based rearrangement yielding lanosterol (Fig. 1.1). A nineteen-step reaction sequence then transforms lanosterol to cholesterol which is a C₂₇-body (Gaylor, 2002). Functionally seen, steroids belong to the lipid class.

According to the nomenclature of steroids (IUPAC-IUB, 1989), the carbon atom numbering and ring letters follow the scheme shown in Figure 1.2. Steroids are defined as structures derived from cyclopentaphenanthrene and often possess two methyl groups

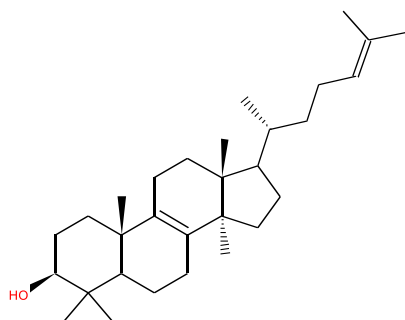


Figure 1.1: Chemical structure of lanosterol.

at C-10 and C-13 and possibly an alkyl side chain at C-17. Members of the steroid subset that bear a hydroxy group at C-3 are called sterols.

The stereochemistry of the steroid's accessory groups is descriptively addressed by assigning to them either the suffix ' α ' or ' β ', depending on whether the group is located below or above the ring plane. The exemplary conformer model of cholesterol (Fig. 1.3) shows that the absolute spatial orientation of the group's covalent bond axis linking it to the respective ring is indeed not homogenous among the α or β -groups due to conformational distortion of the rings. For instance, the methyl groups at C-10 and C-13 and the hydroxy group at C-3 point to different directions in space although they are assigned to the β -orientation.

Counting the number of the potential stereomeric centers of the condensed ring structure displayed in Figure 1.2, yields 4 for the A-ring, 2 for the B-ring, 2 for the C-ring, and 2 for the D-ring which totals to 10. At each of the stereomeric centers a substituent can adopt either the α or the β orientation, yielding a total of 20 possible substitution sites. The permuta-

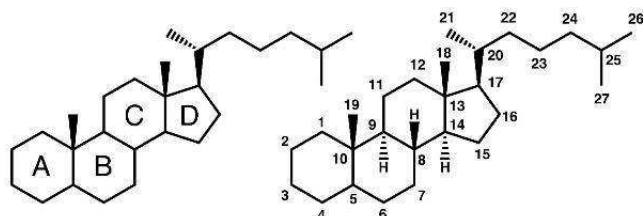


Figure 1.2: Ring designations and atom numbering in steroids.

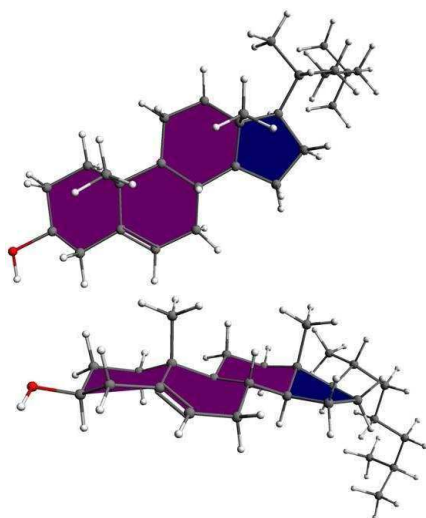


Figure 1.3: An exemplary conformer of cholesterol. The model was rendered using the open-source chemical editor program Avogadro (http://avogadro.openmolecules.net/wiki/Main_Page) combined with openly available 3D-coordinates from a conformer set published by the PubChem project (<http://pubchem.ncbi.nlm.nih.gov/>). The conformer structures in the PubChem database were computed by applying MMFF94 (Merck molecular force field version 94, Halgren (1996)) energy minimization.

tion of k substituents leads to $20!/(20 - k)!$ isomers which results in $20!/17! = 6840$ different structures for already three substituents. This potential variability demonstrates how important stereochemical control is in biosynthesis pathways.

A habit in trivial steroid nomenclature is to name steroids with a double bond between C-5 and C-6 as Δ^5 -steroids. When the double bond is located between C-4 and C-5, the steroids derived thereof are named Δ^4 -steroids.

1.2 Adrenal steroids

The human adrenal is a source of various important steroids. The gland consists of two histologically distinctive regions: the inner medulla and the surrounding cortex whereby the later is composed of three histologically distinguishable layers called the zona glomerulosa, zona fasciculata, and the zona reticularis (Fig. 1.4). Steroid biosynthesis takes place in the adrenal cortex where the responsible enzymes are unequally distributed along the different zones (see for instance Fig. 1.4B) thus yielding a spatially

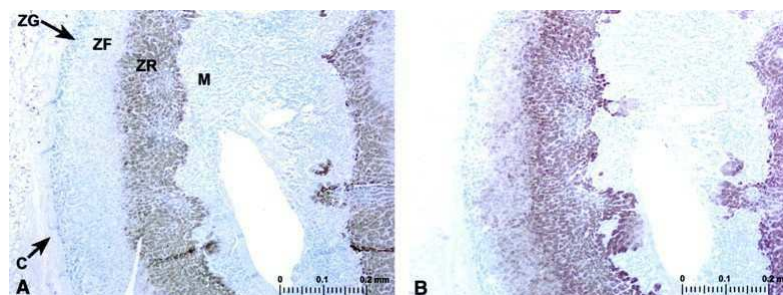


Figure 1.4: Histology of the human adrenal. Immunoreactivity for cytochrome b_5 (A), Immunoreactivity for CYP17A1 (B). C: capsule, ZG: zona glomerulosa, ZF: zona fasciculata, ZR: zona reticularis, M: medula. Image source: Dharia et al. (2004).

varying steroid biosynthesis activity (Ghayee and Auchus, 2007). The most inner part, the zona reticularis, produces C_{19} androgen precursors such as DHEA and its sulfate ($DHEA-SO_3^-$), while the middle layer, the zona fasciculata, and the outer layer, the zona glomerulosa, mainly produce cortisol and aldosterone, respectively.

Figure 1.5 shows a scheme of the main steroidogenic pathways occurring in the adrenal which are catalyzed by cytochrome P450 enzymes and the aldo-keto reductase 3β -hydroxysteroid dehydrogenase (3β -HSD). Note that the steroid biosynthesis system extends over two subcellular compartments, hence, some compounds need to cross at least one membrane. The cytochrome P450 enzymes CYP11A1, CYP11B1, and CYP11B2 are localized in mitochondria while the microsomal CYP17A1 and CYP21A1 are associated with the endoplasmic reticulum (ER). Since the larger part of microsomal P450s topologically faces the cytosol (Poulos and Johnson, 2005), they act as cytosolic enzymes. All shown P450s are able to catalyze more than one reaction. For instance, the mitochondrial CYP11A1 hydroxylates at C-20 and C-22 of cholesterol, the microsomal CYP17A1 catalyzes hydroxylations and bond cleavage reactions, and CYP21A1, CYP11B1,

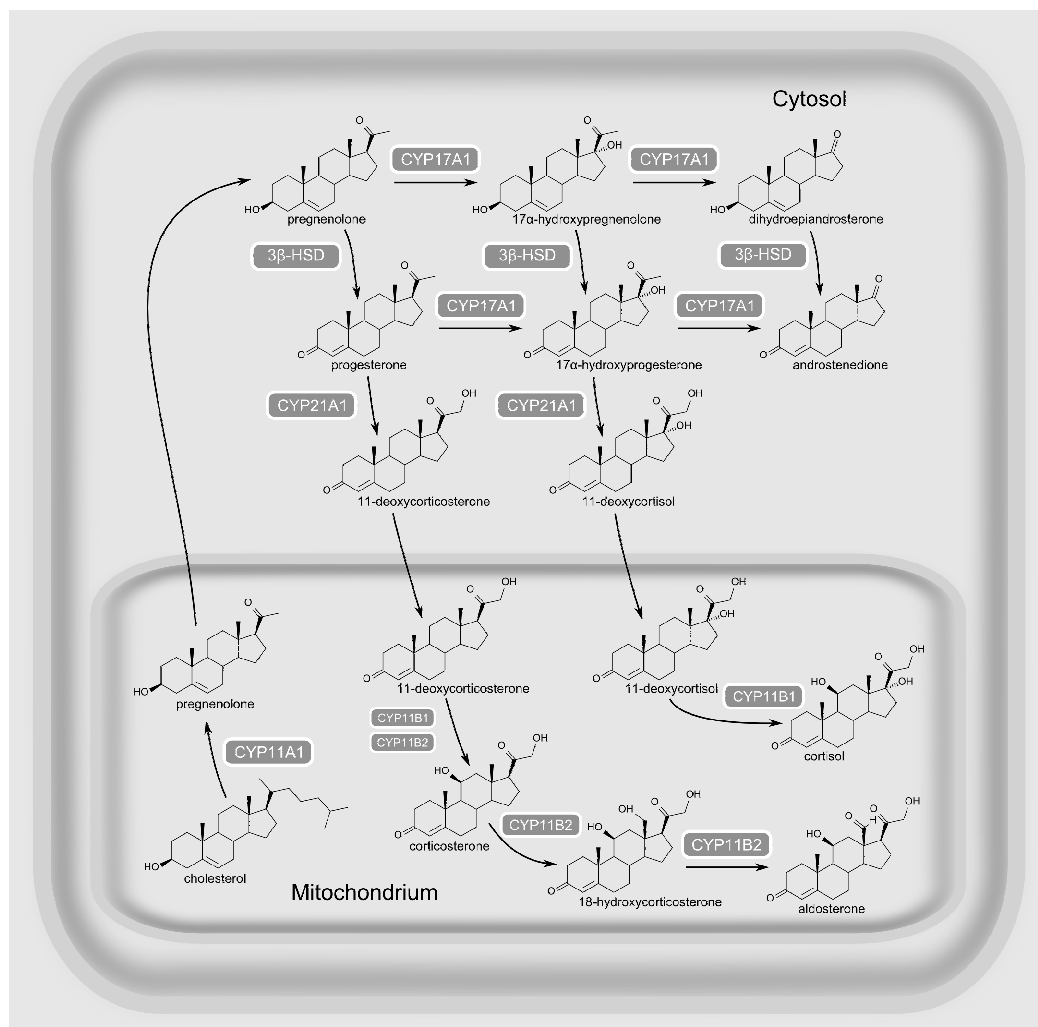


Figure 1.5: Steroid biosynthesis pathways in the human adrenal. CYP: cytochrome P450, HSD: hydroxysteroid dehydrogenase.

and CYP11B2 accept different substrates. Concerning variety, the microsomal CYP17A1 offers the richest repertoire of physiological reactions.

According to Ghayee and Auchus (2007), the metabolic steps required for steroid hormone action in the human body consist of:

1. cholesterol conversion to pregnenolone,

2. pregnenolone conversion into various intermediates,
3. peripheral organ metabolism of precursors and/or physiologically active compounds,
4. specific metabolic effects in the target tissues,
5. degradative metabolism of steroids.

Some crucial steps were omitted in the above enumeration like the availability of cholesterol, the systemic transport of cholesterol by the lipoprotein system (Martini and Pallottini, 2007), and its complex intracellular distribution (Prinz, 2007).

1.3 Clinical aspects of steroid biosynthesis

The specific effects caused by steroid action are often easily observable and it is not surprising that the pivotal studies on the beneficial effects of prescribed adrenal hormones in the treatment of rheumatoid arthritis were gained more than 50 years ago (Hench et al., 1949, 1950). Edward C. Kendall, Tadeus Reichstein, and Philip S. Hench received the 1950 Nobel Prize in Physiology or Medicine for their discoveries related to the hormones of the adrenal cortex. Adverse effects of glucocorticosteroid therapy soon became evident after prolonged treatment periods were applied (Boland and Headley, 1951) and even nowadays a lively debate about dosage and indications is carried out (Bijlsma et al., 2003). However, the need for this class of compounds is still high due to their use in dermatitis and inflammatory chronic diseases (Barnes, 2006). The prominent physiologic function of aldosterone is the increased retention of sodium by the kidney (Freel and Connell, 2004) exerted via the mineralocorticoid receptor. Cortisol is involved in anti-inflammatory actions and complex metabolic effects (Wang, 2005) exerted through the

glucocorticoid receptor. Progesterone acts through the progesterone receptor and plays an important role in the menstrual cycle and pregnancy (Daniel et al., 2009). Its effects were exploited in the form of oral contraceptives. Today, progesterone is substituted by 2nd and 3rd-generation artificial derivatives like levonorgestrel and norgestimate (Kulier et al., 2004). Further important compounds are the C₁₉ steroid DHEA and its sulfate, which are usually transported to the testes or ovaries where sex steroid biosynthesis is accomplished (Ghayee and Auchus, 2007). Their effects on target tissues may become deleterious through the action of testosterone, dihydrotestosterone and estrogen in breast and prostate cancer (Díaz-Chico et al., 2007; Carruba, 2007; Penning and Byrns, 2009).

Clinically seen, the most important P450 member in Figure 1.5 is CYP21A1. Its insufficiency is by far the most common cause of congenital adrenal hyperplasia (CAH). The most life-threatening form is the salt-wasting type leading to dehydration (Ghayee and Auchus, 2007). The symptomatic level of CAH can be correlated with the availability of other hormones which are normally dependent on a physiological CYP21A1 function (see Fig. 1.5). For instance, alterations of sex hormone levels as a consequence of CAH can lead to various phenotypes with respect to sex development and function (Ghayee and Auchus, 2007). The CYP17A1-deficiency is another cause of CAH with an often similarly complex phenotypic appearance while a CYP11B1-deficiency leads to CAH with distinctly increased androgen levels (Ghayee and Auchus, 2007). Overproduction of steroid hormones is known to occur in case of adrenal adenoma and carcinoma or nodular hyperplasia. Classic examples for steroid overproduction disorders are Cushing's syndrome (hyperadrenocorticism, cortisol overproduction) and Conn's syndrome (hyperaldosteronism, aldosterone overproduction) showing some phenotypic symptoms similar to CAH.

The CYP11B subfamily might become interesting as a pharmacological target because of the existence of hyperaldosteronism or hypercorticism syndromes. Although potential inhibitors might play a role here, a couple of reasons hamper the commercial breakthrough of such a strategy. First, a frequently encountered type of hypercorticism is the iatrogenic form caused by the treatment of life-threatening illnesses, such as asthma, rheumatoid arthritis, systemic lupus, inflammatory bowel disease, some allergies, and others (Hopkins and Leinung, 2005); a fact known since many years (Kelly et al., 1972). Second, even in case of adrenocortical tumors it seems that the human CYP17A1 might be the main determinant of the Cushing syndrome (Enberg et al., 2009). Third, due to the mentioned similarity between CYP11B1 and CYP11B2, the screening for a specific inhibitor for one of the isoforms is difficult. However, the link between certain heart diseases and aldosterone levels might generate additional appeal in the near future to be concerned with the human CYP11B subfamily (Bureik et al., 2002a). In fact, the pharmacological concept of a P450 inhibitor drug was already successfully demonstrated for CYP19A1 in the treatment of estrogen-dependent or adrenocortical cancer (Schuster and Bernhardt, 2007).

Due to the effectiveness of their biological activity exerted at low doses, steroids and steroid-derived drugs continue to be valuable pharmacological substances. Bureik and Bernhardt (2007) state that the total steroid world market is probably worth more than 10 billion \$. For instance, several business reports issued during the last years estimate the corticosteroid market for pain, the topical treatment of dermatitis, and intranasal inhalation to be worth more than one billion \$ alone.¹ In the US, a double digit

¹<http://www.ticker.com/Annualreport/CNCT/CNCT-2001.pdf>,
http://www.advertisersservices.com/portfolio/11_November_PP.pdf,
<http://www.ticker.com/Annualreport/ABPI/ABPI-2005.pdf>,
<http://actonpharmaceuticals.com/files/news272/PR2010-01-05.pdf>,
<http://www.domainvc.com/PDF/SkinMedica%20FDA%20approval%20Desonate.pdf>

million number of corticosteroid containing nasal inhalations are prescribed per year.² Moreover, recent internet surveys summarized by Kraska et al. (2010) point towards the existence of hundreds of active websites selling anabolic androgenic steroids at black market prices at least one order of magnitude higher than for the licit compound. Hence, the world steroid black market may account for one billion \$ alone (Baron et al., 2007).

1.4 Steroid synthesis

Steroids were described more than 70 years ago by Heinrich O. Wieland (Nobel prize 1927) and Adolf O. R. Windhaus (Nobel prize 1928). Edward A. Doisy and Adolf F. Butenandt independently isolated estrone, the first steroid hormone to be ever purified, from the urine of pregnant women in 1929 (Nicolaou and Montagnon, 2008). Due to the beneficial therapeutic effects of steroids, an ever increasing demand for steroids arose. The isolation and purification as well as the production of steroids soon became an active field of research.

1.4.1 Biocatalysis by P450s

It took 30 more years after their discovery until the nature of one of the major enzyme groups involved in steroidogenesis was resolved. Initially, Klingenberg (1958) discovered the cellular pigments that showed a differential absorption at 450 nm when complexed with carbon monoxide in the reduced state. Omura and Sato (1962) characterized some properties of these particular cell pigments which, due to their carbon monoxide differential spectrum, were provisionally designated as 'P450s'. Finally, Estrobrook et al. (1963) could demonstrate that P450s were terminal

²<http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/PediatricAdvisoryCommittee/UCM204802.pdf>

oxidases involved in the adrenal steroid biosynthesis.

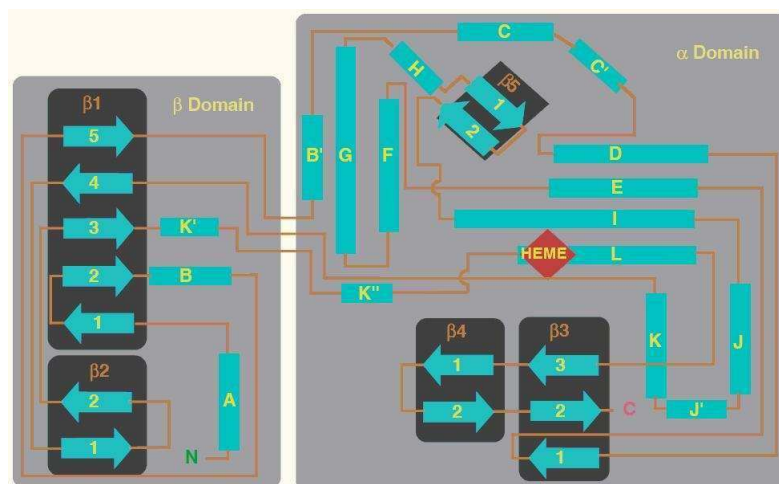
Soon, it became apparent that P450s co-radiated with populations during evolution with at least one representative in every major branch of the tree of life. Therefore, a uniform nomenclature system of the form

$$\boxed{\text{CYP } n \text{ S } m}$$

was set up where 'CYP' stands for 'cytochrome P450', n is the family integer, 'S' being one or two letters designating the subfamily, and m is the integer counter assigned to the individual P450 enzyme (Nelson, 2009). Unfortunately, the name cytochrome was rather inattentively assigned to P450s and, even if they could fit into the cytochrome b class, they are by no means electron transfer proteins but **terminal** monooxygenases (IUB, 1992). In the advent of faster and more reliable DNA sequencing techniques the P450 nomenclature was heavily based on protein sequence identity criteria. The arbitrary chosen 40 % or more identity classifies P450s as belonging to one family while a 55 % identity clusters them into subfamilies (Nelson, 2006). However, the thresholds are now rather regarded as a rule of thumb and phylogenetic clustering, which better reflects evolutionary order, led to the establishment of P450 clans (Nelson, 2006). To date, a total of more than 11000 P450 sequences are known (including variants and pseudogenes) which are spread among 196 bacterial, 12 archaean, 459 fungal, 62 protist, 126 plant, and 120 animal families (Nelson, 2009). The nearly ubiquitous CYP51 (lanosterol 14 α -demethylase) might be the common ancestor to all existing eukaryotic P450s (Nelson, 1999).

A query against the protein data bank³ results in more than 250 X-ray diffraction-gained spatial structures of P450s. In terms of secondary/tertiary structure, P450s reflect their common ancestry. Most P450s are composed of 4 β -sheets (β_5 is variable) and

³<http://www.rcsb.org/pdb/home/home.do>



(A)



(B)

Figure 1.6: Topology and structure of P450s. Panel A shows a topological secondary structure map taken from Peterson and Graham (1998). Rectangles represent helices, arrows β sheets, and lines are the connecting 'random' coils. Panel B displays a recently published CYP51 structure (Chen et al., 2009) complexed with an inhibitor (not shown here). The rendering was done using the KING molecular viewer available from <http://kinemage.biochem.duke.edu/software/king.php>. Secondary structures are indicated by capital letters (α -helices) and the Greek ' β ' (β -strands). The asterisks denote the start and end of protein strand discontinuation due to ambiguous diffraction signals.

13 α -helices (Peterson and Graham, 1998) and follow a topological pattern similar to the one depicted in Figure 1.6A. Figure 1.6B shows an exemplary 3D-structure of the *Mycobacterium tubercu-*

lois CYP51 (Chen et al., 2009). The structural core of P450s consists of the α -helices D, E, I, L, and α -helices J and K (Peterson and Graham, 1998), which are labeled in Figure 1.6B. Helix K contains the absolute conserved motif GXXA which is thought to stabilize the core structure while helix L contains the absolutely required cysteine residue that acts as the fifth iron ligand. Helices I and L contact the porphyrin-heme b prosthetic group while some residues in the B and the I helices may contact the substrate (Guengerich, 2001). Substrate-protein interaction presumably takes place at the substrate-recognition-sequences that were postulated more than 25 years ago by Gotoh (1992) based on sequence alignments. Their role is increasingly supported by experimental data (Guengerich, 2001).

With respect to their chemical composition P450s belong to the metalloproteins. The iron atom is coordinated by the four pyrrole nitrogen atoms of a heme B and the above mentioned, highly conserved cysteine residue of the protein backbone. In the sense of coordination chemistry, ligands to the iron atom form the first coordination shell also designated as a bioinorganic motif (Degtyarenko, 2000). The bioinorganic motif of P450s is denoted as heme-thiolate motif and can be written as



with the iron being shown in the ferric state. The sixth ligand to the ferric iron can be a weak ligand like a hydroxyl group from an adjacent amino acid residue or even water (Kumaki and Nebert, 1978). For instance, carbon monoxide acts as the sixth ligand of the first coordination shell in the reaction leading to the carbon monoxide difference spectrum while in the general case of biocatalysis oxygen plays this role. According to ligand field theory, iron ($[\text{Ar}] 3d^6 4s^2$) in the ferric ($[\text{Ar}] 3d^5$) and ferrous ($[\text{Ar}] 3d^6$) state can undergo a low-spin/high-spin transition upon binding of a sixth ligand due to energy splitting of the 3d orbital in the octa-

hedral complex (Cotton and Wilson, 1976). The high-spin state of the ferrous iron consists of 4 unpaired electrons distributed along two different energy levels of molecular orbitals (in contrast to the low-spin or spin-paired state). The extent to which energy splitting occurs depends on the nature of the ligand; however, a high-spin iron is not necessarily required for catalysis (Guengerich, 2001). Strong ligands like the CN^- -ion usually lead to low-spin states.

The reaction scheme shown in Figure 1.7 illustrates the presumed catalytic mechanism of P450s (see Guengerich (2001) and Guengerich (2007) for further information). Although this scheme was extensively used throughout the literature, it should not be seen as committing in explaining P450 function. It offers neither universality nor certainty in most of its parts, however, it is a good starting point to understand P450 action. The cycle starts with iron being in the ferric state and the substrate binding to the

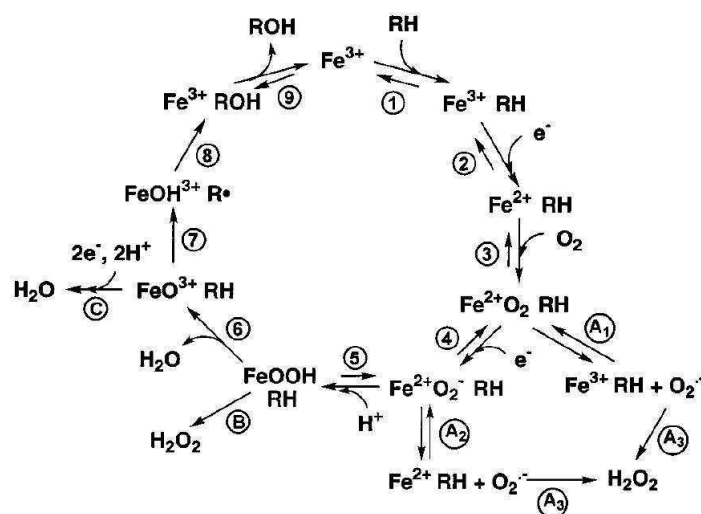
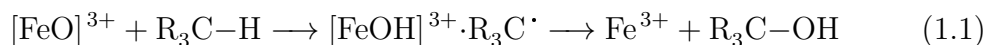


Figure 1.7: Hypothetical P450 reaction cycle leading to the oxygenation of a substrate atom. Reactions denoted with a number are regarded as productive steps while reactions denoted with a letter (including A1, A2, and A3) are seen as abortive exit paths. R: atom or group within the substrate molecule, RH: substrate, image source: Guengerich (2001).

P450 (step 1). The iron atom can but needs not to perform the low-spin/high-spin conversion at this moment. Step 2 comprises the reduction to the ferrous state by an electron transferred from NADH or NADPH via an electron transfer protein (see below). Step 1 is not required for step 2 to occur and is shown before just to illustrate that it is faster. Moreover, substrate binding and dissociation can take place at different steps of the catalytic cycle (Isin and Guengerich, 2008). Ferrous iron binds molecular oxygen (step 3) and then receives the second electron (step 4) from an electron transfer protein which can be different from the one used in step 2. Beyond step 3, the characterization of the individual enzymatic states is uncertain and controversial (Guengerich, 2001, 2007). The $[\text{Fe}^{2+}\text{O}_2]$ as well as the $[\text{Fe}^{2+}\text{O}_2]^-$ complexes are unstable and generate superoxide anions and finally H_2O_2 through the abortive steps A1 to A3. In case A1 to A3 do not take place, a proton attaches to the oxygen radical forming a ferrous peroxo-complex ($[\text{Fe}^{2+}\text{OOH}]$, step 5) which either generates H_2O_2 (step B) or undergoes dehydration to yield a $[\text{FeO}]^{3+}$ -complex (step 6). The precise electronic configuration of $[\text{FeO}]^{3+}$ is not known although some authors denote this as $\text{Fe}^{\text{V}}=\text{O}$ (Guengerich, 2001). The homologous peroxidase complex rather points toward Fe^{4+} and a one-electron deficiency delocalized in the porphyrin-ring system (Guengerich, 2001). The $[\text{FeO}]^{3+}$ -complex is apparently used in most P450 reactions. Further reduction and addition of protons can lead to water formation in the abortive step C. However, abstraction of one hydrogen from the substrate generates a substrate radical (step 7) which then receives the hydroxyl group in step 8 to finally dissociate from the enzyme in step 9. Guengerich (2001) offers a general mechanism of the form



that rationalizes the following observed P450 reactions:

- carbon hydroxylation,

- heteroatom release (N, S, and O-dealkylations),
- heteroatom oxygenation,
- epoxidation/group migration.

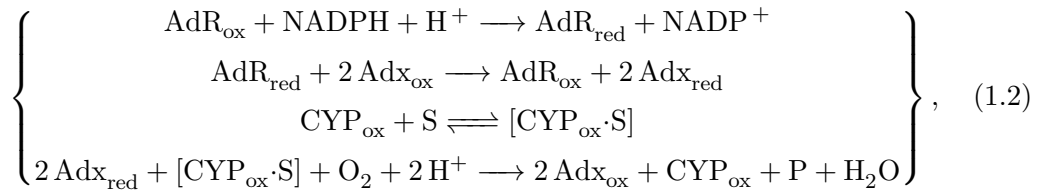
Dehydrogenations share the hydrogen abstraction step with carbon hydroxylations, however, they terminate by stripping off a vicinal proton thus yielding a double bond. There are additional reactions catalyzed by P450s that cannot be explained by Equation 1.1. Indeed, the chemical properties of the different P450 iron complexes are diverse (Mansuy, 1998). For instance, Fe^{3+} is not quite reactive but due to the cysteinyl ligand is able to transfer an electron to peroxides, e.g., prostacyclin synthase (CYP8A1) without involvement of any electron transfer proteins. The ferrous P450 is more electron rich and can participate in reactions like

- reduction of nitro compounds,
- N-oxide reduction,
- reductive dehalogenation.

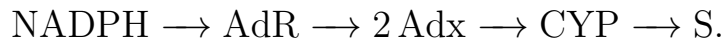
Eventually, the $[\text{Fe}^{2+}\text{O}_2]^-$ -complex is nucleophilic and may react with substances containing an electrophilic center (Mansuy, 1998). An important role of the protonated peroxo complex $[\text{Fe}^{2+}\text{OOH}]$ is in the carbon-carbon bond cleavage reactions of CYP17A1 and CYP19A1 (Ahmed and Owen, 1998). Technically, both the relative position of the substrate to the heme-iron as well as the various iron species may be responsible for the chemical variety of P450 catalysis. It seems that a coherent, general P450 mechanism seems to be far away nowadays, nevertheless, the mechanistic variety is what makes P450s so exciting.

Except for the Fe^{3+} -catalyzers (see above), the electron supply of P450s must be assured during catalysis. With one exception

where electrons are derived from pyruvate or coenzyme A, all P450-systems utilize NADH or NADPH as electron donors that are linked to the terminal P450 by electron transfer proteins (Hanemann et al., 2007). To date, 10 different P450 electron transfer classes can be distinguished of which the highest diversity is expectably found in bacteria. All P450s shown in Figure 1.5 can be assigned to two classes. The mitochondrial CYP11A1, CYP11B1, and CYP11B2 are embedded in a class I electron transfer system consisting of NADPH, adrenodoxin reductase (AdR), and adrenodoxin (Adx) which performs the following reactions

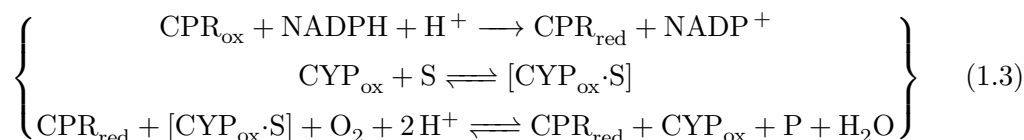


where S denotes the substrate and P the product. Using the simplified redox scheme, the above equation might be rewritten as



Hereby, the substrate association to the P450 is shown before Adx binding, however, substrates may bind after reduction by Adx. The 57 kDa AdR, which is loosely associated with the inner mitochondrial membrane, possesses a bipartite FMN-FAD reaction center enabling it to accept two electrons from NADPH. The electrons are then sequentially transferred to the one-electron storing 14 kDa Adx which shuttles them towards the terminal P450. To simplify the above reaction, the two-electron transfer was shown as one reaction. The interaction between AdR and Adx occurs mainly through electrostatics comprising basic residues located on AdR and acidic residues near the Fe_2S_2 -center on Adx (Miller, 2005). Both CYP21A1 and CYP17A1 are embedded in a class II electron transfer system consisting of NADPH and cytochrome

P450 reductase (CPR) that catalyze



Again, substrate binding is shown before reduction by CPR which appears realistic in light of K_d -values in the micromolar range (Nakajin et al., 1981; Kominami et al., 1988). The vectorial electron flow in class II systems can be simplified as



Electrons from NADPH are transferred by the 77 kDa CPR via electrostatic interaction to the P450 and finally to the substrate (Miller, 2005). It is not quite clear whether CYP17A1 might require accessory electron transfer through cytochrome b_5 in order to carry out the 17/20-lyase reaction (Auchus et al., 1998).

The steroidal products of P450s are predominantly accumulated in tissues and body fluids of higher eukaryotes. In the early days of steroid discovery, biological raw materials were hence often the starting point for tedious purification processes. For instance, less than 70 mg of estriol were isolated from 200 L of human pregnancy urine by Marrian (1930), 100 mg of equilin were isolated from 7 t of mare urine by A. Girard 1932 (Cartland and Meyer, 1935), 25 mg of estradiol was purified from 4 t of fresh sow ovaries by Edward A. Doisy in 1936 (Nicolau and Montagnon, 2008) while in 1934 20 mg of progesterone could be isolated from 625 kg of sow ovaries by the German-based Schering Laboratories (Nicolau and Montagnon, 2008). The above numbers demonstrate that an enormous amount of work was necessary to yield amazingly low quantities of pure steroids. This fact renders any direct isolation attempt of steroids from naturally occurring biomass sources at kilogram scale commercially unattractive. However, the great demand for steroids that followed the pivotal studies by

Hench et al. (1949) forced the implementation of commercially viable production processes.

1.4.2 Chemical synthesis

The first total synthesis of a steroid (equilenin) was achieved as early as 1939 by Bachmann, Cole, and Wilds while estrone was synthesized in 1948 and non-aromatic steroids followed during the 1950s (Hirschmann, 1992). The total synthesis of cortisone by Woodward et al. (1951) marks an important milestone since it was shown that even stereochemically demanding structures could be built from relatively simple compounds. Later on, during the 1970s, the first biomimetic synthesis approach was applied to the synthesis of progesterone by William S. Johnson while Peter K. C. Vollhardt showed an astonishingly simple total synthesis of estrone (Nicolaou and Montagnon, 2008).

Despite these academic achievements, commercially viable steroid syntheses never relied on a total synthesis approach alone but rather started with naturally occurring structures and followed a partial synthesis scheme. One of the most influential approaches was designed for progesterone by Russell E. Marker starting from diosgenin (Fig. 1.8A) which can be isolated from the Mexican yam (*Dioscorea mexicana*). Carl Djerassi managed to partially synthesize cortisone from diosgenin in 1951, the same year in which Woodward had presented his total synthesis of the same steroid (Nicolaou and Montagnon, 2008). Cortisone could first be commercially provided by the Merck Laboratories in 1944 using dioxycholic acid (Fig. 1.8B) and derivatives thereof as starting material (Hirschmann, 1992). The Schering corporation soon followed with a partial synthesis of cortisone using bile acid as raw material and extended their portfolio to prednisone, prednisolone, and dexamethasone but increasingly included biotransformation steps in their processes (Herzog and Oliveto, 1992). The Schering

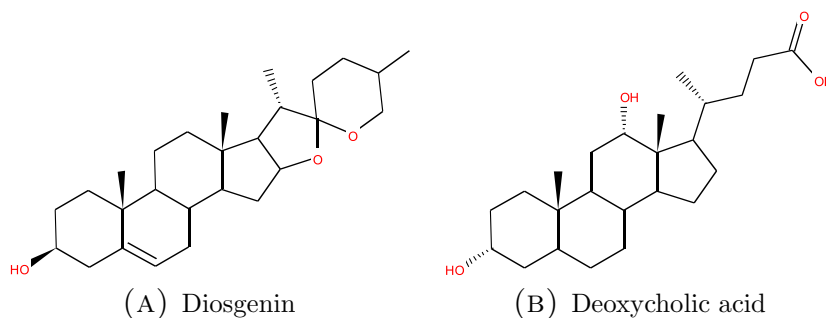


Figure 1.8: Starting structures for commercially viable partial syntheses of steroids.

corporation was second to Merck to introduce steroid formulations on the market and cortisone acetate was already produced at around 100 kg per month by that time (Herzog and Oliveto, 1992).

As mentioned, biotransformation of steroids became increasingly interesting for the industry. Some industrially useful microorganisms are certain corynebacteria (21-deacetylation), *Flavobacterium dehydrogenans* (11 β -deacetylation), *Pestalotia foedans*, and *Glomerella cingulata* (11 α -hydroxylation) as reported by Herzog and Oliveto (1992). At the Squibb Institute for Medical Research, the microbial hydroxylation of progesterone was published in 1952 (Fried, 1992). During the same year, chemists from Upjohn demonstrated the 11 β -hydroxylation of 11-deoxycortisol by *Streptomyces fradiae* (Colingsworth et al., 1953), the 11 α -hydroxylation of progesterone by *Rhizopus arrhizus*, and the partial synthesis of triamcinolone using *Arthrobacter roseochromogenus* (Fried, 1992). Another commercially useful biotransformation step is the 11 β -hydroxylation of steroids by *Curvularia lunata* of which even the responsible P450 could be purified (Suzuki et al., 1993). More information on microbial steroid biotransformations can be found in Bureik and Bernhardt (2007). It is obvious that microbial biotransformation steps found their way into commercially viable partial syntheses right from the beginning of

steroid production by chemical and pharmaceutical companies.

1.5 The rationale for this work

1.5.1 Focus on recombinant whole-cell biotransformation

It often comes to oversimplification when balancing the pros and cons of a biotechnological process. Some arguments often mentioned in favor of microbial biotransformation are

- stereospecificity due to the enzymatic nature of catalysis,
- energy-saving because the process can be carried out at moderate temperatures and pressures,
- low safety-risk processes as a consequence of the above points and due to the absence of potentially harmful organic substances,
- environmentally more friendly due to the above mentioned points.

Each of the above arguments turns out to be very differently assessed when thoroughly analyzed.

For instance, stereospecificity was previously shown to be sometimes controllable in organic synthesis by different means. One opportunity is the biomimetic approach simultaneously giving rise to several correct stereomeric carbon centers. This technique was firstly applied by Johnson et al. (1971) to synthesize progesterone at 12 % efficiency and cortisol acetate at considerable yields (Johnson et al., 1980).

The energy-saving argument needs a thorough analysis with the particular process at hand—a rather utopian situation, since industrial processes are rarely disclosed. Nevertheless, many reasons can lead to considerable energy consumption by a microbial

process: sterilization of bioreactors and accessory piping and devices, cooling of bioreactors, agitation energy and energy demand of the system's pumps, motors, and actuators. It should not be forgotten that many chemical steroid syntheses, either complete or partial, can be carried out at atmospheric pressure (see for instance all references in Sec. 1.4.2).

Indeed, the safety risk of a microbial process appears greatly reduced compared to classical organic synthesis techniques due to reduced use of potentially harmful chemical compounds. However, biological safety measures may be neglected only in case a GRAS⁴ status has been granted to the production organism and are otherwise of concern. Steroids possess a lower solubility in aqueous solutions than in organic solvents implying the use of organic solvent-based liquid-liquid extraction (LLE) procedures in order to isolate the steroid from the culture broth. Potential sideproducts formed by the biomass may necessitate further separation leading to increased use of solvents. Eventually, the hazard risk associated with this techniques is for sure considerable while the risk owing to high pressure vapor sterilization may likewise not be neglected.

If the above paragraphs obscure the usefulness of biotransformation, the question is why then focus on it? The answer starts with a simple fact. An important aspect of industrial processes is to be profitable. If costs do steadily rise during operation, an equally steady growing need to further increase the process efficiency will arise. Since we deal here with processes that are run over decades, development has been carried out over several years, often during runtime, resulting in a virtually evolutionary optimization. It is obviously only attractive to completely replace an established process by a complete redesign in case of a high efficiency gain, therefore, often partial steps are subjected to further optimization. This work targets one of the steps employed

⁴generally regarded as safe

in the partial synthesis of cortisol being a microbial process carrying out the 11β -hydroxylation of 11-deoxycortisol. Hence, the reason why this work focused on biotransformation was that the manufacturer wished to potentially replace an existing biotransformation step and required the use of existing equipment.

In light of heterologous expression techniques, a possible replacement of the existing microbial process may be the use of a genetically modified organism that is able to carry out the desired reaction more efficiently. This approach would elegantly circumvent the tedious screening for organisms that possess a natural 11β -hydroxylase. Despite a remarkable body of work carried out using expressed human CYP11B1 and CYP11B2 in mammalian cell culture systems (Bureik et al., 2002a), only few hosts were available at the time this work was initiated that would lend themselves for industrial processes.

A promising step was, however, reported by Dumas et al. (1996) who transformed a baker's yeast strain with genetic elements carrying the cDNAs of the bovine CYP11B1 and its redox partner Adx. This genetic operation rendered the yeast strain capable of performing 11β -hydroxylation of 11-deoxycortisol and 11-deoxycorticosterone. Some key points worth noting are that the authors replaced the native targeting signal of the P450 by the yeast's COXVI signal while the coexpression of adrenodoxin was found to be crucial for the detection of CYP11B1 activity.

1.5.2 Use of human P450s and fission yeast

Reviewing the sequencing status of genomes⁵ reveals that none of the industrially used microorganisms (*Streptomyces fradiae*, *Cunninghamella blakesleeana*, and *Curvularia lunata*) used in the partial synthesis of cortisol was sequenced in 2001. A consequence

⁵http://www.genomenewsnetwork.org/resources/sequenced_genomes/genome_guide_index.shtml

of lacking sequence knowledge is that the naturally occurring 11β -hydroxylases of the mentioned organisms cannot be easily exploited because the unknown cDNA sequence of the responsible enzyme cannot be accessed by homology searches. Moreover, overexpression in the natural host is equally inaccessible due to non-existent molecular biology tools. One must assume that controlling expression, activity, and hence, the efficiency of the conversion reaction is restricted to classical bioprocess optimization techniques applied to the natural host. To circumvent the need to employ such laborious methods, this work focuses on the use of recombinant fission yeast strains expressing P450 species known to perform the required reactions.

Fission yeast is a single-celled free-living organism belonging to the phylum of sac fungi (Tab. 1.1) with common features like fungal-type cell wall, closed mitosis, ascus-type sporangium, ascomycetes-like mode of sex determination and others. Yeasts in a broader sense are a diverse group of organisms and, for instance, baker’s yeast belongs to the subphylum Saccharomycotina while fission yeast to the subphylum Taphrinomycotina. During the advent of DNA sequencing it became clear that fission yeast could be classified as equally distant from mammalia as from *Saccharomyces cerevisiae* (Sipiczki, 1995; Wood et al., 2002). The determination of the divergency point between the subphylums of *Saccha-*

Table 1.1: Taxonomy of fission yeast

Taxon	Name
kingdom	Fungi
phylum	Ascomycota
subphylum	Taphrinomycotina
class	Schizosaccharomycetes
order	Schizosaccharomycetales
family	Schizosaccharomycetaceae
genus	<i>Schizosaccharomyces</i>
species	<i>Schizosaccharomyces pombe</i>

romyces (Saccharomycotina) and *Schizosaccharomces* (Taphrinomycotina) is somewhat vague due to missing sequences from certain member species in the Taphrinomycotina (Sipiczki, 2000). Therefore, the separation of fission and baker's yeast must have occurred between more than 300 million years (Sipiczki, 2000) and nearly one billion years ago (Heckman et al., 2001; Hedges, 2002), while the separation of fission yeast from Metazoa and plants took place around 1.6 billion years ago (Heckman et al., 2001; Wood et al., 2002).

Concerning cell cycle (Russell and Nurse, 1986), RNAi-silencing (White and Allshire, 2008), centromere organization and control (Allshire and Karpen, 2008), splicing machinery (Kaufer and Potashkin, 2000), RNA polymerase III (Huang and Maraia, 2001), transcription (Remacle et al., 1997), protein kinases (Bimbo et al., 2005), and many other cellular processes and structures, fission yeast shows more similarity to higher eukaryotes than baker's yeast. That does not necessarily mean that fission yeast is evolutionary closer to mammals but that there is a chance that some cellular features will be closer to mammals than in other yeasts. Fission yeast also shows a more mammalian-like post-translational modification pattern and was successfully used for the heterologous production of proteins in a considerable number of studies (Giga-Hama and Kumagai, 1997). Moreover, the expression of microsomal P450s was demonstrated in three articles using members of the CYP2C-subfamily (Yamazaki et al., 1993; Yasumori et al., 1999; Takanashi et al., 2000), albeit for in vitro studies. With a fully sequenced genome (Wood et al., 2002) that was already available online in 2001 and well established methods (Forsburg and Rhind, 2006), fission yeast offers good access to molecular biotechnology. Additionally, culturing and fermentation techniques might be easily implemented due to its yeast nature and do not have to be designed from scratch. An additional benefit of fission yeast might also be the low endogenous

P450 background of comprising only two species (Wood et al., 2002), CYP51 (sterol 14 α -demethylase) and CYP61 (sterol Δ^{22} -desaturase). Noteworthy, baker's yeast possesses an additional P450 that resembles the *Aspergillus nidulans* CYP56B1 (Goffeau et al., 1996).

However, one of the most influential factors that led to the use of fission yeast in this work was the successful functional expression of human CYP11B2, a very close relative of the cortisol-producing CYP11B1, in fission yeast (Bureik et al., 2002b). Hereby, several key factors could be identified that evaluated fission yeast as a suitable host for the heterologous expression of human CYP11B1. First, Western blot development of DISC-SDS-PAGE-separated protein lysates isolated from mitochondria indicated correct targeting of the native CYP11B2. In this way, it could be presumed from the Western band pattern that the targeting sequence could be partially processed by the host. The second and more important point was the evidence for the 11 β -hydroxylase activity of CYP11B2 towards both 11-deoxycorticosterone and 11-deoxycortisol. Third, a fission yeast electron transfer protein called Etp1 was identified in this study that rendered the human CYP11B2 activity self-sufficient. Additionally, the coexpression of the human CYP11B1 and Etp1 led to an increased conversion rate. In contrast, Adx coexpression was crucial in baker's yeast in order to record significant bovine CYP11B1 activities (Dumas et al., 1996).

As shown in Figure 1.5, the human CYP11B1 is located in mitochondria of adrenal cortex cells (Fig. 1.4) and catalyzes the conversion 11-deoxycortisol to cortisol as well as the conversion of 11-deoxycorticosterone to corticosterone (Bureik et al., 2002a). The human CYP11B1 and CYP11B2 are very similar enzymes sharing 95 % identity. Both are synthesized as 503 aa long precursors including the 24 aa long mitochondrial targeting sequence which is excised upon translocation to the inner mitochondrial

membrane (Bureik et al., 2002a). Human CYP11B1 is only functional when being part of a class I electron transfer system comprising of AdR and the Fe₂S₂-ferredoxin Adx (Hannemann et al., 2007). At the time this work was initiated, the human CYP11B1 was functionally expressed only in mammalian cell lines like CHO, COS-7, and V79 (Denner et al., 1995a,b) while the heterologous expression of rat CYP11B1 in *Escherichia coli* resulted in very low yields (Nonaka et al., 1998).

The ability of the fission yeast to serve as a host for the expression of recombinant human P450s was further examined in this work by including the expression of two microsomal, steroidogenic P450s. The human CYP21A1 catalyzes the immediate upstream reactions leading to the substrates of CYP11B1 and CYP11B2 (see Fig. 1.5). The human CYP17A1 reactions are more complex. First, it can hydroxylate the C-17 of either progesterone or pregnenolone to yield 17 α -hydroxyprogesterone and 17 α -hydroxypregnenolone, respectively (see Fig. 1.5). Furthermore, both 17-hydroxylated steroids can be subjected to a carbon-carbon bond cleavage between C-17 and C-20 which is catalyzed by the CYP17A1 via a mechanism that is similar to the CYP19A1 reaction (Ahmed and Owen, 1998). However, the human form of CYP17A1 is known to efficiently carry out bond cleavage mainly on Δ^5 -steroids (Miller, 2005).

The successful heterologous expression of the human CYP11B2 (Bureik et al., 2002a) and the previously shown functional expression of human microsomal P450s in fission yeast (Yamazaki et al., 1993; Yasumori et al., 1999; Takanashi et al., 2000) not only justified the use of fission yeast as a host but also the use of human enzymes as catalysts. In fact, it is the combination of fission yeast and human P450s that pointed towards a successful system.

1.6 Aims of this work

The main objective of this work was the construction of a functional human CYP11B1 expressing fission yeast strain and the phenotypic enhancement of the 11β -hydroxylase activity on a laboratory scale. Since at the start of this work only a combination of thin-layer chromatography-based analytics using radioactive substrates was available, the development of suitable HPLC-based analysis methods was also required. Furthermore, as a preparation for the coexpression of further components of the class I electron transfer system used by CYP11B1 (see reaction system 1.2), the setup of a suitable whole-cell biotransformation assay format and the comparison of recombinant fission yeast strains expressing different CYP11B1 variants was intended. To test fission yeast's ability to carry out whole-cell biotransformations when used as a host for human microsomal P450s, the study was extended to functionally express the human CYP17A1 and CYP21A1, to develop suitable analytical methods, and to setup a suitable laboratory-scale biotransformation assay for the particular activities.

Chapter 2

Discussion

2.1 Functional expression of human CYP11B1 in fission yeast

Within the context of this work, some preliminary studies were conducted on the expression of a human CYP11B1 variant that showed three amino acid mutations compared to the previously published sequence (Mornet et al., 1989). The F494C substitution was described by Kawamoto et al. (1990) while the L52M and the I78V substitutions by Denner et al. (1995a,b); Böttner et al. (1996, 1998); Cao and Bernhardt (1999); Bechtel et al. (2002). Later on, the mentioned positions within the human CYP11B1 were substituted by the originally described amino acids (Mornet et al., 1989; Kawamoto et al., 1990) resulting in four human CYP11B1 expressers (see Hakki et al. (2008) and Tab. 2.1). Additionally, Table 2.1 also shows the used parent strains, the CYP11B2 expressor MB164, and both microsomal P450 expressors.

Table 2.1: Fission yeast strains used in this work.

Strain	Genotype	CYP11B1 variant	Reference
MB163 ¹	<i>h⁻ ura4-D18</i>	none	NCYC
MB175	<i>h⁻ ade6-M210 leu1-32 ura4-D18 his3-Δ1</i>	none	Burke and Gould (1994)
MB164	<i>h⁻ ura4-D18 leu1::pCAD1-CYP11B1</i>	<i>h₁CYP11B2</i>	Bureik et al. (2002b)
SZ1	<i>h⁻ ura4-D18 leu1::pCAD1-CYP11B1</i>	<i>h₁CYP11B1</i>	Dragan et al. (2005)
SZ52 ²	<i>h⁻ ura4-D18 leu1::pCAD1-CYP11B1^{I78V}</i>	<i>h₁CYP11B1^{I78V}</i>	Hakki et al. (2008)
SZ78 ³	<i>h⁻ ura4-D18 leu1::pCAD1-CYP11B1^{L52M}</i>	<i>h₁CYP11B1^{L52M}</i>	Hakki et al. (2008)
CAD1	<i>h⁻ ura4-D18 leu1::pCAD1-CYP11B1^{L52MI78V}</i>	<i>h₁CYP11B1^{L52MI78V}</i>	Bureik et al. (2004)
CAD8	<i>h⁻ ade6-M210 leu1-32 ura4-D18 his3-Δ1 pNMT1-hCYP17</i>	<i>h₁CYP17A1</i>	Dragan et al. (2006b)
CAD18	<i>h⁻ ade6-M210 leu1-32 ura4-D18 his3-Δ1 pNMT1-hCYP21</i>	<i>h₁CYP21A1</i>	Dragan et al. (2006a)

1: NCYC2036

2: originally named SZM52L

3: originally named SZ1V78I

2.1.1 The human CYP11B1 is expressed and correctly localized in fission yeast cells

The pINT5 derivative, pCAD1, was successfully used to integrate the *Pnmt1-hCYP11B1* expression cassette into the *leu1* locus on chromosome II of parent strain NCYC2036. All *h₁CYP11B1* proteins were expressed as fusion proteins with a C-terminal *his₆* followed by a Pk-tag (Dragan et al., 2005). After identifying correct integrands by replica plating onto EMM medium containing phloxine but lacking leucine, clones were chosen for further characterization. Under fully induced expression conditions, the constructed strains CAD1, SZ52, SZ78, and SZ1 showed a wildtype-like phenotype and a significantly slower growth when compared to the parent strain NCYC2036 (data not shown). The slower growth kinetics was also observed with the *h₁CYP11B2* expressor MB164 (Bureik et al., 2002b) and might be interpreted as a consequence of mitochondrial P450 overexpression.

The theoretical average mass of the expressed CYP11B1 is composed of the mature 55 kDa stemming from the structural part plus the 2 kDa stemming from the fused *his₆* and Pk-tags yielding 57 kDa. Denaturing DISC-SDS-PAGE of mitochondrial lysates of the respective strains combined with α -Pk/ α -rabbit antibody-based Western detection of tagged proteins revealed a

signal with a molecular mass between 48 kDa and 62 kDa for both ${}_h\text{CYP11B1}^{\text{L52MI78V}}$ (not shown) and ${}_h\text{CYP11B1}$ (Dragan et al., 2005). The apparent molecular masses of both CYP11B1 variants were hence in agreement with the above calculated mass and also with published data on ${}_h\text{CYP11B1}$ expression in COS-1 cells (Böttner et al., 1998) and expression of the 98 % identical ${}_h\text{CYP11B2}$ in fission yeast (Bureik et al., 2002b).

Obviously, similar to the expression of CYP11B2, the native CYP11B1 presequence could correctly target the protein to the fission yeast mitochondria irrespective of the CYP11B1 variant. Since no additional signals were observed in the gel lanes containing the separated mitochondrial proteins following Western blot analysis (${}_h\text{CYP11B1}^{\text{L52MI78V}}$ analysis not shown, ${}_h\text{CYP11B1}$ analysis in Dragan et al. (2005)), post-translational processing of the CYP11B1 variants seemed to be terminated at the time of cell disruption. Remarkably, this complete processing could not be observed in mitochondrial lysates of the CYP11B2 expressor MB164 (Bureik et al., 2002b).

2.1.2 Fission yeast strains expressing the human enzyme CYP11B1 convert 11-deoxycortisol to cortisol in vivo

The initial 11-deoxycortisol biotransformations were performed with the ${}_h\text{CYP11B1}^{\text{L52MI78V}}$ expressor CAD1 and analyzed using thin-layer chromatography (TLC) as well as high-pressure liquid chromatography (HPLC) revealing the formation of a specific product in CAD1 samples (data not shown). The TLC analysis of the 11-deoxycortisol biotransformation showed a product that migrated at the same distance as the cortisol reference. Additionally, the use of ^3H -labeled 11-deoxycortisol as a substrate confirmed the presence of the radioactive signal within the spot of the product in TLC analysis combined with autoradiography

(Bureik et al., 2004). These results were confirmed by HPLC analysis where the formation of a compound was observed that showed the same retention time and the same spectroscopic properties determined by a diode array detector (DAD) as the cortisol reference (not shown). Thus, the detected product was specific to the biotransformation by CAD1 and was not present in samples stemming from the parent strain NCYC2036. After performing site-directed mutagenesis on the ${}_h\text{CYP11B1}^{\text{L52MI78V}}$ cDNA sequence to yield ${}_h\text{CYP11B1}$, the initial results obtained using strain CAD1 could be confirmed by strain SZ1 with both methods TLC combined with autoradiography (Dragan et al., 2005) as well as HPLC (not shown). Taken together, the results mentioned in this paragraph demonstrated the successful biotransformation of 11-deoxycortisol to cortisol by fission yeast strains expressing ${}_h\text{CYP11B1}$. The significant activity of the recombinant fission yeast strains demonstrated that the presence of the C-terminal his_6 and the Pk-tag consisting of 16 additional amino acids did neither abolish substrate binding nor electron transfer to the P450.

The analysis of the biotransformation samples revealed the presence of a byproduct derived from 11-deoxycortisol that could be readily detected by TLC analysis using visual fluorescence quenching, autoradiography, and HPLC analysis (data not shown). This byproduct appeared in samples of strain CAD1, SZ1, and the parent strain NCYC2036, indicating a potential side reaction taking place in the host. One explanation for the appearance of the byproducts is the loss of the C-4,C-3-enone group of the 11-deoxycortisol's A-ring which could be, in principle, catalyzed by the previously described Δ^4 -reductase combined with a 3α -HSD activity (Pajic et al., 1999). However, this pathway seems improbable since it would rather lead to a significant absorption shift towards lower wavelengths due to the ketone group still present at C-20. A more probable explanation for the presence

of a byproduct might be the endogenous 20α -HSD activity (Pajic et al., 1999). Albeit the 20α -HSD activity of fission yeast was demonstrated using progesterone and 20α -hydroxyprogesterone, reduction of other 20-oxo-steroids cannot be ruled out. A possible activity towards the C-20,C-17-ketol group is, for instance, supported by reports where a bacterial (Krafft and Hylemon, 1989) and a mammalian 20α -HSD (Sato et al., 1972) are indeed able to reduce the keto group at C-20 of 11-deoxycortisol and 17α -hydroxyprogesterone, respectively.

2.1.3 Space-time yield on cortisol

As mentioned in the introduction, one intention of this study was to elucidate possible replacements of actual industrial steroid bioprocesses. Hence, in order to better assess the ability of the developed systems, the parameter space-time yield ($\langle CR \rangle$) given in $\mu\text{M d}^{-1}$, which is usually used in reactor process engineering, was applied to the analysis of steroid bioconversion data. There are different ways of calculating the space-time yield. One possibility is to determine the product concentration $c(t)$ at a given time t , usually given in days, and then to calculate

$$\langle CR \rangle = \frac{c(t)}{t}. \quad (2.1)$$

However, since the formation rate of cortisol and other steroidal products was not constant during the biotransformation period as observed in this work, the space-time yield between consecutive sampling times defined as

$$\langle CR \rangle_{t^*,t} = \frac{c_t - c_{t^*}}{t - t^*} \quad (2.2)$$

with t, t^* in days and $t > t^*$ was also used. The $\langle CR \rangle$ -function can be identified as the first derivative of $c(t)$ for $t - t^* \rightarrow 0$. As might be presumed when regarding the unit of $\langle CR \rangle$, no normalization to biomass-related parameters was made yielding a

parameter that is highly dependent on the biotransformation conditions. Therefore, a set of biotransformation conditions was set as standard conditions consisting of a biotransformation period of 72 h, a cell suspension volume of 10 mL, a substrate concentration of 1 mM added from a 40 mM ethanolic stock solution, an incubation temperature of 30 °C, an agitation of 300 rpm, and a terminal HPLC analysis.

The average space-time yield on cortisol over a 72 h biotransformation period ($\langle CR \rangle_{0,3}$) for the ${}_h\text{CYP11B1}^{\text{L52MI78V}}$ expressor CAD1 under standard conditions was $9.6 \pm 0.4 \mu\text{M d}^{-1}$, while SZ52 showed $6.7 \pm 0.5 \mu\text{M d}^{-1}$, SZ78 $47 \pm 3 \mu\text{M d}^{-1}$, and SZ1 $55 \pm 3 \mu\text{M d}^{-1}$ (not shown). Thereby, the kinetics of the cortisol formation was nearly linear until $t = 48$ h where a slight rate decrease occurred (not shown). These data demonstrated the significant adverse effect of the I78V mutation within CYP11B1. The resulting activity relation between the fission yeast strains expressing different ${}_h\text{CYP11B1}$ variants could be confirmed in an assay using 100 μM 11-deoxycortisol (Hakki et al., 2008). According to a three-dimensional homology model of ${}_h\text{CYP11B1}$, the amino acid residue at the position 52 lies at the surface of the enzyme and hence, is regarded as a minor influence when subjected to substitution. In contrast, position 78 is located near the substrate binding site and is expected to exert more influence on activity (Hakki et al., 2008). This theoretical thoughts should be regarded with caution due to the numerous approximations made during the generation of a homology model. The only valid statement that can be made on firm ground to date is that the 78 position was unexpected to play such a role in activity.

The dependence of $\langle CR \rangle$ on the biotransformation conditions can be readily seen when regarding the results of Appel et al. (2005) where a rate of approximately $100 \mu\text{M d}^{-1}$ was determined using a growing CAD1 culture and HPLC analysis. Moreover, using concentrated cell suspensions of strain SZ1 express-

ing ${}_h\text{CYP11B1}$ and 5 mM 11-deoxycortisol concentrations yielded space-time yields of $200 \mu\text{M d}^{-1}$ as analyzed by TLC (Dragan et al., 2005). In contrast, the yield on cortisol using the bovine CYP11B1 expressed in baker's yeast was less than 2 % of a 100 mg L^{-1} (approximately $290 \mu\text{M}$) initial concentration (Dumas et al., 1996), which translates to roughly $5.8 \mu\text{M}$ of cortisol. This amount was measured in a cell suspension growing from $2 \times 10^7 \text{ cells mL}^{-1}$ to $6 \times 10^8 \text{ cells mL}^{-1}$ during a 72 h incubation period yielding $\langle CR \rangle_{0,3} = 1.9 \mu\text{M d}^{-1}$. Thus, the $\langle CR \rangle_{0,3}$ value of SZ1 measured under standard conditions was at least 28 times higher. Apparently, the presence of Adx and AdR was essential in baker's yeast in order to reach a space-time yield of about $12 \mu\text{M d}^{-1}$ under the described conditions (Dumas et al., 1996). Nevertheless, the baker's yeast figure is still more than 4.5 times and 16 times lower when compared to SZ1 under standard (not shown) and optimized conditions (Dragan et al., 2005). Remarkably, this was achieved in absence of Adx coexpression using a roughly three times lower cell density than used with baker's yeast.¹ Of course, the reason for the differences between fission and baker's yeast are difficult to explain due to the complex experimental and physiological differences between the two systems. Whether fission yeast might be evolutionary less derived than baker's and hence, would offer a more convenient cellular environment for the expression of human CYP11B1 will be addressed in the next section.

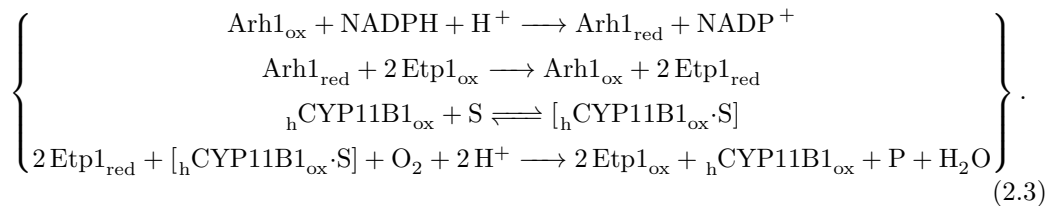
2.1.4 Fission yeast electronically sustains mitochondrial P450 reactions

Obviously, in fission yeast the expressed ${}_h\text{CYP11B1}$ species could couple to an endogenous electron transfer, as was expected from previous findings reported by Bureik et al. (2002b). However,

¹Assuming linear growth during the bioconversion period of baker's yeast.

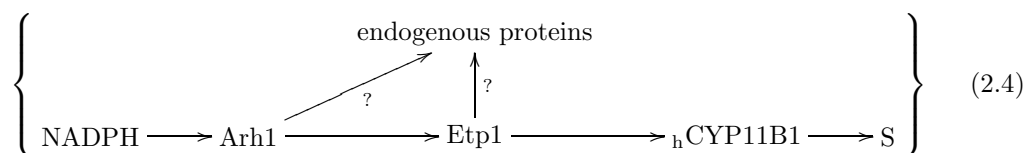
the presence of an electron transfer chain serving human, mitochondrial P450s seemed peculiar in an organism whose lineage departed from the main mammalian branch around 1.6 billion years ago (Heckman et al., 2001; Wood et al., 2002) and even more so in absence of an endogenous mitochondrial P450. Fortunately, past and recent work significantly contributed to the elucidation of the electron transfer chain’s nature.

The mitochondrially localized ferredoxin Etp1 was presented as a candidate with a high probability of playing the role of Adx based on protein sequence homology, preliminary electron transfer experiments, and overexpression studies performed in fission yeast (Bureik et al., 2002b). Later on, further experimental data demonstrated that electron transfer on bovine CYP11B1 could be sustained by this protein (Schiffler et al., 2004; Ewen et al., 2008). Moreover, the existence of a putative Etp1 reductase (SPBC3B8.01c, Arh1) was also first postulated by Bureik et al. (2002b) and later on experimentally characterized and confirmed by Ewen et al. (2008). The statistical analysis of the fission yeast Arh1 protein sequence revealed a much higher identity to bovine and human AdR than to the Arh1 sequence of baker’s yeast (Ewen et al., 2008). Including the results of the mentioned studies, the putative redox system responsible for the observed 11 β -hydroxylase activity in fission yeast strains expressing human, mitochondrial CYP11B1 can be described by:



At least in baker’s yeast, it could be shown that mitochondrial ferredoxin and ferredoxin reductase participate in heme biosynthesis (Barros et al., 2002) which might also be the native functions of Arh1 and Etp1 in fission yeast. Consequently, since ad-

ditional electron sinks cannot be ruled out, host-related electron sinks must be considered according to



by use of the vectorial electron flow symbolism. These findings imply that the ability to transfer electrons from NADPH to a mitochondrially localized P450 might have evolved before and independent of the presence of the terminal P450. As mentioned above, the ramification of the fission yeast and the mammalian lineages occurred more than 1 billion years ago but fission yeast seemed to retain more mammalian-like structures and processes than the more derived baker’s yeast (Sipiczki, 2000). This could explain why in baker’s yeast the presence of $_{\text{b}}\text{Adx}$ was crucial in order to yield detectable $_{\text{b}}\text{CYP11B1}$ activity (Dumas et al., 1996) while in fission yeast the endogenous systems were able to sustain $_{\text{h}}\text{CYP11B1}$ activity. However, since $_{\text{b}}\text{CYP11B1}$ was not expressed in fission yeast, it cannot be ruled out that $_{\text{h}}\text{CYP11B1}$ cooperates more efficient with Etp1 than $_{\text{b}}\text{CYP11B1}$.

2.1.5 The human CYP11B1 and CYP11B2 show different kinetic properties when expressed in fission yeast

Since the nearly identical fission yeast strains MB164 (expresses $_{\text{h}}\text{CYP11B2}$) and SZ1 (expresses $_{\text{h}}\text{CYP11B1}$) were available, it was interesting to compare the in vivo activities of the expressed enzymes. Due to the previous studies using the mutated form of $_{\text{h}}\text{CYP11B1}$ (Denner et al., 1995a,b; Böttner et al., 1996, 1998; Cao and Bernhardt, 1999; Bechtel et al., 2002), the activity comparison included also the $_{\text{h}}\text{CYP11B1}^{\text{L52MI78V}}$ expressing fission strain CAD1.

When assayed under standard conditions, strain MB164 expressing ${}_h\text{CYP11B2}$ showed a nearly 8 and 60 times lower space-time yield on cortisol ($\langle CR \rangle_{0,1} = 1 \pm 0.6 \mu\text{M d}^{-1}$, not shown) when compared to the value of strain CAD1 ($\langle CR \rangle_{0,1} = 7.8 \pm 0.6 \mu\text{M d}^{-1}$, not shown) and strain SZ1 ($\langle CR \rangle_{0,1} = 60 \pm 3 \mu\text{M d}^{-1}$, not shown), respectively. Regarding the overall 72 h biotransformation period, the space-time yield of MB164 was still slightly more than 3 times and 18 times lower than for CAD1 and SZ1. These relations are more or less consistent with the approximately twenty times higher 11-deoxycortisol conversion rate of ${}_h\text{CYP11B1}^{\text{I78V}}$ compared to ${}_h\text{CYP11B2}$ when expressed in COS-1 cells (Böttner et al., 1996). Although being described as the preferred substrate, the measured space-time yield of MB164 on corticosterone during the initial 24 h biotransformation period ($\langle CR \rangle_{0,1} = 0.9 \pm 0.4 \mu\text{M d}^{-1}$, not shown) was 30 times lower than for SZ1 ($\langle CR \rangle_{0,1} = 30 \pm 2 \mu\text{M d}^{-1}$, not shown) while for the average space-time yield ($\langle CR \rangle_{0,3}$) this ratio reduced to 18 (data not shown). No values were gained for the 11-deoxycorticosterone biotransformation by CAD1. The observed reduction in the $\langle CR \rangle_{0,3}$ ratio between MB164 and SZ1 for both substrates 11-deoxycorticosterone and 11-deoxycortisol was phenomenologically caused by the four-fold activity increase of MB164 during the last 48 h of biotransformation when compared to the initial 24 h. In contrast, the SZ1 activity was more or less constant throughout the whole biotransformation period.

Peculiarly, during IC_{50} determination studies, MB164 could convert approximately 20 % of 100 nM 11-deoxycorticosterone within a period of 8 h (Bureik et al., 2004) while CAD1 needed 24 h to achieve similar results with 11-deoxycortisol (not shown). This means that under relatively low substrate concentrations the turnover of ${}_h\text{CYP11B2}$ was higher than the turnover shown by ${}_h\text{CYP11B1}^{\text{L52MI78V}}$.

These apparently contradictory results could be resolved by

enzyme kinetics alone. If conversion velocities of the CYP11B1 and CYP11B2 systems would be described by pseudo-Michaelis-Menten kinetics dependent on the substrate concentration, the activity differences observed in this work and by Böttner et al. (1996) could be easily explained if

$$K_{1/2}(\text{CYP11B1}) > K_{1/2}(\text{CYP11B2}), \quad (2.5)$$

$$v_{\max}(\text{CYP11B1}) > v_{\max}(\text{CYP11B2}) \quad (2.6)$$

would hold. This, in turn, implies the existence of a specific substrate concentration c^* which relates to the assayed substrate concentration c_S as

$$c_S \begin{cases} < c^* & \Rightarrow v(\text{CYP11B1}) < v(\text{CYP11B2}), \\ = c^* & \Rightarrow v(\text{CYP11B1}) = v(\text{CYP11B2}), \\ > c^* & \Rightarrow v(\text{CYP11B1}) > v(\text{CYP11B2}). \end{cases} \quad (2.7)$$

Böttner et al. (1996) reported previously that the conversion rates of CYP11B1 and CYP11B2 could not be kinetically distinguished at an initial 11-deoxycortisol concentration of 250 nM but first at 5 μM . Thus, the presumed substrate concentration c^* at which turnover identity would hold between ${}_h\text{CYP11B1}^{\text{L52MI78V}}$ and ${}_h\text{CYP11B2}$ might have been near the reported 250 nM in COS-1 cells. Later on, for the case $c_S > c^*$ the correctness of the above relations could be demonstrated in a direct comparison between MB164 and SZ1 using substrate concentrations between 10 μM to 1 mM (data not shown). However, different kinetic parameters of the P450s were surely not the only factors that could contribute to conversion rate differences between CYP11B1 and CYP11B2.

For instance, the efficiency of the presequence targeting and processing upon mitochondrial import might be different between

the two enzymes. Actually, no direct and systematic comparison was made to address this.

The C-terminal tags fused to the CYP11Bs present another difference. A his_6 followed by a Pk-tag were fused to all expressed CYP11B1 variants while solely a his_6 tag was fused to the CYP11B2 expressed by MB164 (Bureik et al., 2002b). At least in *E. coli*, a C-terminal his_6 tag was described as advantageous concerning proteolytic degradation of expressed human enzymes (Nonaka et al., 1998). However, whether the presence of the additional C-terminal Pk-tag might be beneficial for the CYP11B1 variants remains uncertain.

Electron delivery by the host seems to be guaranteed for both enzymes but the specific electron transfer rates might differ. According to Equation 2.3, the operative interaction between the host and the expressed enzymes has to take place between Etp1 and the P450s. The share of this interaction to the macroscopically measured space-time yield might only be assessed using in vitro systems.

The expression of CYP11B1 and CYP11B2 differentially affected the growth of the host. While the growth rate constant of MB164 was nearly three-fold lower compared to the parent strain, CAD1 showed an only two-fold lower figure (data not shown). This might be a sign of more deleterious physiologic effects caused by ${}_h\text{CYP11B2}$ compared to ${}_h\text{CYP11B1}$ that might explain the observed biotransformation differences between MB164, and CAD1 or SZ1.

Eventually, it should be kept in mind that the saturation concentration of 11-deoxycorticosterone is around 100 μM in EMM while the value for 11-deoxycortisol is one order of magnitude higher (data not shown). Although Equation 2.5 and Equation 2.7 will not lose their validity per se, the portion of the relation describing the kinetics for substrate concentration greater than 100 μM will be heavily influenced by the different solubility

of the substrates. Indeed, steroid solubility would offer an even simpler explanation for the observed effects. Consequently, the space-time yield values obtained using 1 mM substrate concentrations will include the solubility effect and might not be linked to any of the above mentioned factors.

2.1.6 Application of CYP11B1 expressing fission yeast strains for inhibition studies

Strain CAD1 was used during the discovery process of selective CYP11B1 inhibitors as part of an already existing assay design (Bureik et al., 2004) which was further developed during this work. Most of the discussion dealing with the applicability of fission yeast strains expressing human mitochondrial P450s for the discovery of selective CYP11B1 and CYP11B2 inhibitors was already dealt with by Bureik et al. (2004). The facts that will be stressed here will focus more on the technical side of the system's development.

During the 24 h incubation period of strain CAD1, a passive gas exchange pathway between cell suspension and atmosphere was required in order to obtain measurable conversion rates. This could be achieved in a very simple way by modifying conventional 1.5 mL tubes (Fig. 2.1). Since conical 200 μ L pipette tips were used to assure gas exchange, their lengths determined the diameter of the pathway and hence, also the flow resistance. As could be demonstrated, the length of the gas exchange pathway exerted considerable control over the reaction rate (data not shown). At the first glance the successful application of the tip-tubes might appear self-evident because oxygen appears as an educt in the CYP11B1 reaction (Eq. 1.2). However, a more important parameter is the oxygen consumption rate of the host which leads to rapid depletion of the dissolved oxygen as was shown with considerable lower cell density suspensions of strain MB175 (Dragan

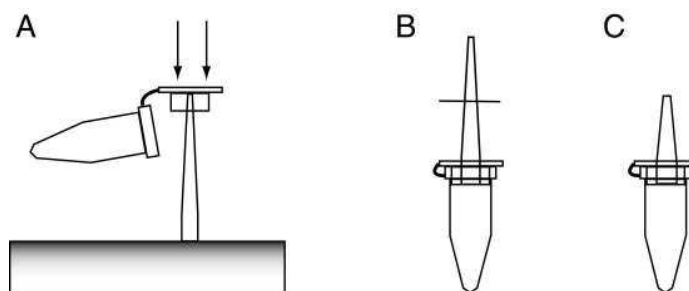


Figure 2.1: Modification of a 1.5 mL tube to yield a tip-tube. The exhaust channel is made by piercing the cap of a 1.5 mL reaction tube with a 200 µL pipette tip (A). To avoid plastic material blocking the air pathway, the pipette tip was cut at the indicated line (B) to finally yield a tip-tube (C).

et al., 2006a). Consequently, a potential oxygen competition between host and recombinant processes might arise that could be effectively prevented using tip-tubes. Another important factor observed during the incubation was that gas exchange pathways were extremely beneficial for the dissipation of pressure built up in the previously tightly closed tubes by carbon dioxide evolution.

2.2 Functional expression of the microsomal human P450s CYP17A1 and CYP21A1 in fission yeast

2.2.1 Expression of microsomal P450s

Transformation of fission yeast strain MB175 with the plasmids pNMT1-hCYP17 and pNMT1-hCYP21 resulted in the new strains CAD8 and CAD18 (see Tab. 2.1). The new strains grew significantly slower than the parent strain MB175 but did not show any phenotypic alterations (data not shown). Protein extraction samples gained after inducing protein expression in absence of thiamine showed specific signals when probed with an α -Pk antibody that were not present in the protein samples of the parent strain

(Dragan et al., 2006a,b). The apparent molecular masses of the signals were found to be in good agreement with the theoretically calculated masses of the tagged proteins being 59 kDa and 58 kDa for the ${}_h\text{CYP17A1}$ and the ${}_h\text{CYP21A1}$, respectively. Moreover, a good agreement regarding the ${}_h\text{CYP17A1}$ molecular mass was also observed when compared to other heterologous expression systems like *E. coli* (Imai et al., 1993; Grigoryev et al., 1999), baker's yeast (Auchus et al., 1998), and *Pichia pastoris* (Kolar et al., 2007). Similar results were observed for the ${}_h\text{CYP21A1}$ molecular mass when expressed in COS-1 cells (Tusie-Luna et al., 1990), *E. coli* (Hsu et al., 1999), and baker's yeast (Hsu et al., 1996).

When regarding the gel lane loaded with CAD8 cell lysate, a distinctive band pattern that specifically reacted with the α -Pk antibody was detected. Since no similar signals were observed in the lane loaded with cell lysate stemming from the parent strain, it can be concluded that the fragments showing lower apparent molecular masses than 59 kDa were C-terminal fragments derived from the expressed ${}_h\text{CYP17A1}$. Although very often the Western analysis was not shown in research articles using heterologous ${}_h\text{CYP17A1}$ expression while others paid little attention to the fact, similar electrophoretic findings were often observed in bacterial (Imai et al., 1993), baker's yeast (Auchus et al., 1998; Gupta et al., 2001; Costa-Santos et al., 2004), and *Pichia pastoris* (Kolar et al., 2007) lysates. The observed degradation behavior of ${}_h\text{CYP17}$ is worth noting since it apparently took place in various heterologous expression systems.

2.2.2 Human CYP17A1 and CYP21A1 are functionally expressed in fission yeast

The recombinant fission yeast strain CAD8 expressing ${}_h\text{CYP17A1}$ converts progesterone to 17α -hydroxyprogesterone and to 16α -

hydroxyprogesterone as well as pregnenolone to 17 α -hydroxypregnenolone (Dragan et al., 2006b). The steroid 16 α -hydroxyprogesterone could not be detected in HPLC chromatograms of cholesterol oxidase-treated pregnenolone bioconversion samples, suggesting no hydroxylation of pregnenolone at C-16. Moreover, no androstenedione signal was observed in any sample regardless whether oxidase treatment was involved or not, which strongly points towards a missing 17,20-lyase activity. The measured space-time-yields for the first 24 h of biotransformation were calculated as $\langle CR \rangle_{0,1} = 176 \pm 13 \mu\text{M d}^{-1}$ and $\langle CR \rangle_{0,1} = 83 \pm 9 \mu\text{M d}^{-1}$ for 17 α -hydroxyprogesterone and 16 α -hydroxyprogesterone, respectively (Dragan et al., 2006b). Thereby, the $\langle CR \rangle$ -values reached a plateau before $t = 72$ h.

The observed relative space-time yield magnitudes of the detected products were similar to biotransformation results obtained using COS-1 cells expressing ${}_h\text{CYP17A1}$ (Swart et al., 1993). Hitherto, the metabolite 16 α -hydroxyprogesterone was regularly encountered in ${}_h\text{CYP17A1}$ activity assays, for instance when expressed in *E. coli* (Ehmer et al., 2000), baker's yeast (Auchus et al., 1998), and *Pichia pastoris* (Kolar et al., 2007). It seems that this pathway might be physiologically active according to data gained using fetal adrenal and adult testis microsomes (Swart et al., 1993). The missing C-16 hydroxylation of pregnenolone was also in accordance with data gained by Swart et al. (1993).

The 17,20-lyase activity of ${}_h\text{CYP17A1}$ seems to be mainly exerted on 17 α -hydroxypregnenolone but not on 17 α -hydroxyprogesterone (Auchus et al., 1998; Gilep et al., 2003). However, the use of purified, recombinant ${}_h\text{CYP17A1}$ produced in *E. coli* demonstrated a more than 100-fold lower 17,20-lyase activity towards 17 α -hydroxypregnenolone compared to the 17-hydroxylase activity towards pregnenolone (Imai et al., 1993). A relatively low 17,20-lyase activity towards 17 α -hydroxypregnenolone was also confirmed using JM109 *E. coli* whole cells expressing solely

${}_h\text{CYP17A1}$ (Sagara et al., 1993). Moreover, baker's yeast microsomes containing coexpressed ${}_h\text{CYP17A1}$ and reductase showed a lower 17,20-lyase activity towards 17α -hydroxyprogesterone compared to the activity towards 17α -hydroxypregnenolone (Auchus et al., 1998). Common to all mentioned studies where 17,20-lyase activity was detected was the use of C-17 hydroxylated steroids as substrates. Since this was not the case in this work, at least the weak 17,20-lyase activity towards 17α -hydroxypregnenolone might have been suppressed by pregnenolone being present in excess.

The direct comparison of the ${}_h\text{CYP17A1}$ metabolite space-time yields determined in this work and most published studies was difficult due to the fact that in vitro studies usually report rates normalized to the P450 content. On the other hand, rates were often gained under relatively low substrate concentrations leading to figures far below v_{\max} . One report stated that *E. coli* cells expressing ${}_b\text{CYP17A1}$ and ${}_r\text{CPR}$ were able to convert $50\ \mu\text{M}$ progesterone at initial rates of up to $10^4\ \mu\text{M d}^{-1}$ (Shet et al., 1997). However, this remarkable figure could be sustained for only a relatively short period of 5 min. Moreover, activity in presence of $200\ \mu\text{M}$ progesterone decreased apparently in an exponential fashion yielding rates of around $1200\ \mu\text{M d}^{-1}$ when referred to a 30 min incubation period. Hence, it can be concluded that the *E. coli* system could be hardly applied in the large-scale production of metabolites under the published conditions. More comparable assay conditions were applied to the conversion of progesterone by a recombinant baker's yeast strain expressing ${}_b\text{CYP17A1}$. This study reported rates around $240\ \mu\text{M d}^{-1}$ (Degryse et al., 1999), which were similar to values obtained in this work (Dragan et al., 2006b).

The fission yeast strain CAD18 was able to convert progesterone to 11-deoxycorticosterone and 17α -hydroxyprogesterone to 11-deoxycortisol in vivo (Dragan et al., 2006a). Thereby, the

measured space-time yields during the first 24 h of the biotransformation period were $\langle CR \rangle_{0,1} = 21 \pm 1 \mu\text{M d}^{-1}$ and $\langle CR \rangle_{0,1} = 259 \pm 9 \mu\text{M d}^{-1}$ for 11-deoxycorticosterone and 11-deoxycortisol, respectively (Dragan et al., 2006a). The later reaction was carried out at a rate roughly one order of magnitude higher than the former. This finding correlates well with data from reports using recombinant $_{\text{h}}\text{CYP21A1}$ expressed in baker's yeast, where the apparent hydroxylation rate of 17α -hydroxyprogesterone was also considerably higher than that for progesterone (Wu et al., 1991). The use of $_{\text{b}}\text{CYP21A1}$ expressed in COS-1 cells similarly yielded lower rates for the progesterone conversion (Lorence et al., 1989), while purified recombinant $_{\text{h}}\text{CYP21A1}$ displayed the same preferences as well (Hsu et al., 1999). Since most authors did not focus on whole-cell biotransformation, no space-time yield comparison could be reliably performed. As was the case with studies dealing with $_{\text{h}}\text{CYP17A1}$, most reports in which $_{\text{h}}\text{CYP21A1}$ reactions were measured worked at lower substrate concentrations than used in this work.

2.2.3 Application of CYP17A1 and CYP21A1 expressing fission yeast strains for inhibition studies

Using both CAD8 and CAD18, it was possible to set up an IC_{50} screening system under similar conditions as used for strain CAD1 or MB164 (Dragan et al., 2006b). As in the case of the mitochondrial P450 expressing fission yeast strains, the specific discussion dealing with the particular inhibitors is dealt with in the article. It shall be mentioned here, however, that the main result in this study was to determine that $_{\text{h}}\text{CYP21A1}$ should be carefully regarded when developing $_{\text{h}}\text{CYP17A1}$ inhibitors due to the relative high resemblance (29 % amino acid identity) between the two enzymes. Thus, as could be shown in the study, cross-inhibition of $_{\text{h}}\text{CYP21A1}$ by $_{\text{h}}\text{CYP17A1}$ inhibitors cannot be ruled out. Dur-

ing the assay design phase, a couple of interesting points worth mentioning surfaced.

First, using relatively low substrate concentrations (100 nM) revealed that the progesterone conversion rate differences between h CYP17A1 and h CYP21A1 expressing strains, usually observed when using 1 mM substrate concentrations, vanished. Similar to the rate difference explanation given for mitochondrial P450s (Sec. 2.1.5), it might be presumed that the kinetics are approaching identity under decreasing substrate concentrations. In contrast to the mitochondrial P450 expressing strains, however, the microsomal expressing fission yeast strains were able to convert 25 % to 40 % of the respective substrates within 15 min of assay time (Dragan et al., 2006b).

Second, frequent sampling revealed the formation of a byproduct. Applying kinetic modeling of the CYP17-system under IC_{50} assay conditions yielded the theoretical model A as the best fit to experimental data with 17α -hydroxyprogesterone being the precursor of the observed byproduct (Dragan et al., 2006b). The correctness of model A might be supported by a study using baker's yeast expressing b CYP17 where 17α -hydroxyprogesterone was converted by an endogenous 20α -HSD to $17\alpha,20\alpha$ -dihydroxyprogesterone (Shkumatov et al., 2002). Since fission yeast possesses a 20α -HSD (Pajic et al., 1999), it is highly probable that the same reaction might take place in fission yeast as well. As mentioned above (see Sec. 2.1.2), 20α -HSDs from other organisms were able to reduce the keto group at C-20 of 17α -hydroxyprogesterone (Sato et al., 1972; Krafft and Hylemon, 1989), so the presumed reduction might occur as in baker's yeast.

Third, it is noticeable that in contrast to the IC_{50} values obtained using microsomal preparations containing h CYP17A1 the values measured using the fission yeast system were generally biased towards higher values (Dragan et al., 2006b). This effect might be explained by the presence of basic nitrogen containing

chemical groups in the structures of the tested inhibitors. Their pH-dependent charge might largely contribute to their ability to cross the fission yeast's plasma membrane. Since microsomal assays are usually carried out at $\text{pH} = 7.4$, nitrogen group containing compounds might be significantly present in their uncharged form while in the acidic fission yeast medium ($\text{pH} = 5.4$) their ratio might be significantly reduced. Charged compounds hardly can pass membranes unless suitable transporter systems are at hand, so the observed differences might be due to pure thermodynamics.

2.2.4 Electronic coupling of microsomal P450s to host systems and its implications

Since hydroxylated metabolites of $_{\text{h}}\text{CYP17A1}$ and $_{\text{h}}\text{CYP217A1}$ substrates were readily detected during biotransformation assays, a viable transfer of electrons to the human enzymes can be assumed to take place in the host. A highly probable primary source of electrons to the P450s is the endogenous fission yeast CPR homologue expressed from the *ccr1* gene (Wood et al., 2002). Although the host's putative CPR was found to share more than 30 % identity to higher mammalian CPR sequences and bears crucial flavine coenzyme binding motifs (Miles, 1992), its exact function was not yet experimentally elucidated. A secondary source of electrons, at least for the $_{\text{h}}\text{CYP17A1}$, might be cytochrome b_5 . However, since no 17,20-lyase activity was observed in this work, the involvement of the host's cytochrome b_5 system including the cytochrome b_5 reductase (CBR) remains questionable.

Often in baker's yeast and also in fission yeast, growing cells induce sterol and desaturated fatty acids syntheses (Koukkou et al., 1993) in order to cope with changing environmental conditions like increasing fermentative ethanol concentrations and hypoxia. In fission yeast, the transcription of the responsible genes was

shown to be upregulated in a sterol regulatory element binding protein (SREBP)-dependent way (Hughes et al., 2005). It is well known that at several steps in the sterol and fatty acid biosynthetic pathway redox reactions are involved that require electron supply. For instance, the fission yeast squalene monooxygenase (SMO), the 22-sterol desaturase (CYP61A1) and the sterol 14 α -demethylase (CYP51A1) depend on reduction equivalents supplied by the CPR of which Rosenfeld et al. (2002) reported that it might be a considerable source of reactive oxygen species (ROS) in baker's yeast. Already more than 40 years ago, data gained with fission yeast demonstrated the existence of a non-respiratory oxygen consumption not linked to glucose consumption (Heslot et al., 1970) which apparently seems to confirm the data published by Rosenfeld et al. (2002).

The expression of microsomal P450s by strains CAD8 and CAD18 led to significantly lowered growth rate constants when compared to the wildtype strain MB175 (data not shown). This observation was assumed to be correlated to the fully induced expression state of *Pnmt1*. As a possible consequence it could be suspected that overexpressed functional P450s are competing for electrons in the host's cellular environment and, therefore, are representing an additional electron sink that interferes with the above mentioned sterol biosynthesis pathway leading to growth retardation. At the same time, this competition might phenomenologically lead to reduced oxygen consumption due to the reduced loss of electrons by the host's CPR. Oxygen measurements as well as ROS generation data gained in this work seems to point towards a view where the presence of substrate significantly reduces oxygen consumption and ROS generation in both strains CAD8 (data not shown) and CAD18 (Dragan et al., 2006a). Moreover, in presence of substrates metabolic flux ratio analysis revealed a relative increase in the TCA cycle flux of strain CAD18 (expresses $_{h}$ CYP21A1) showing approximately the same

relative magnitude as the recorded decrease of the oxygen consumption rate and the amount of generated ROS (Dragan et al., 2006a). These findings might be interpreted as a sign for the competition of intracellular processes for oxygen leading to the observed activity reduction of the respiratory electron transfer systems in favor of the overexpressed microsomal P450s. The experimental data were consistent with the simulation results gained by numerically solving the developed kinetic model (Dragan et al., 2006a). The data showed a similar decrease in oxygen consumption and ROS generation under increasing ${}_h\text{CYP21A1}$ substrate concentrations and, hence, increasing CYP21 activity. In addition, in this model oxygen plays a double role within the P450 systems. On one hand it acts as a terminal electron acceptor while on the other it acts as a cosubstrate in the P450 reaction (see equation system 1.3).

Overall, simulation results were numerically well comparable to the experimentally gained figures concerning product formation rate, oxygen consumption rate, and ROS generation for both CAD8 and CAD18 (data not shown).² To further test the answer of the kinetic model to different parameter inputs, the simulation was extended to comprise two varying factors at the same time (data not shown). Hereby, the increased oxygen consumption rates in presence of ${}_h\text{CYP21A1}$ and absence of substrates were the only deviations from experimental results (data not shown). Nevertheless, the output of the model should be rather seen qualitatively since kinetics can be potentially described by a different set of reaction combinations. It should be also kept in mind that the model only delivered rates displayed by a 'P450 only'-system which were devoid of additional cellular oxygen consumption rates. Hence, the model failed to reproduce the generally

²Comparing the simulation data with experimental values can be accomplished by dividing the simulated figures by 166 in order to yield rate and concentration values referred to one liter of cell suspension. Further reduction of the oxygen consumption rate must be taken into account due to the lower cell density used in the MTP assays (Dragan et al., 2006a).

higher oxygen uptake of the parent strain MB175 although it correctly described the decrease in total oxygen consumption in presence of substrates (data not shown). Moreover, steroid metabolite formation rates were quite consistent with experimentally determined rates within the tested range of substrate concentration (not shown).

A further important constituent of a P450 system is the source of reduction equivalents, NADPH (see equation system 1.3). Looking at the pentose phosphate pathway (PPP) as one possible NADPH-source by metabolic flux ratio analysis revealed no differences between the presence and absence of substrates in case of strain CAD18 (Dragan et al., 2006a). This leads to the conclusion that fission yeast did not upregulate the PPP in order to react on the NADPH demand induced by the P450-dependent biotransformation. Of course, the existence of alternative NADPH sources that were not included in the reaction network of the metabolic flux ratio analysis might have been overlooked. However, at first sight the experimental results conform the simulation results where the consumption of oxygen, the generation of ROS, and of products first reacted to decreasing NADPH concentration at values below 1 μM (not shown). This weak dependence on the NADPH concentration could be experimentally confirmed by Zehentgruber et al. (2010) who recently demonstrated that the intracellular NADPH concentration of around 20 μM kinetically reacted to the presence of a biotransforming P450 system. However, after reaching equilibrium it stabilized at values between 5 μM and 10 μM . Consequently, it could be hypothesized that there might be more room for further improvement of the 17α -hydroxyprogesterone biotransformation rate of strain CAD18. Indeed, Zehentgruber et al. (2010) could readily increase the initial biotransformation rate of 17α -hydroxyprogesterone 6-fold over the value shown by strain CAD18 (Dragan et al., 2006a).

Conclusions

In this work the heterologous and functional expression of the human CYP11B1 could be achieved using fission yeast as a host (Dragan et al., 2005). As was expected from previous work (Bureik et al., 2002b), the human CYP11B1 was correctly localized to the host's mitochondria and received electrons from a present electron transfer chain. Together with a formerly generated fission yeast strain expressing the human CYP11B2 (Bureik et al., 2002b), the fission yeast system could be successfully used for the discovery of a hit compound that showed the selective inhibition of one of the closely related isoforms (Bureik et al., 2004).

A further confirmation for use of fission yeast as a host was achieved by the functional expression of two microsomal P450s, CYP17A1 (Dragan et al., 2006b) and CYP21A1 (Dragan et al., 2006a). Thereby, it could be shown that fission yeast was able to sustain a significant metabolite formation rate during whole-cell biotransformation. A significant increase of the space-time yield of CAD18 could be later achieved by Zehentgruber et al. (2010). The biotransformation data imply the presence of an electron transfer chain in the host that is able to support human microsomal P450s. Moreover, it could be shown that both fission yeast strains could be used for the screening of specific inhibitors (Dragan et al., 2006b). Later studies showed that the coexpression of human CYP21A1 and CPR allowed the application of a CYP21A1 expressing strain to the structural determination of a sports doping agent metabolite (Zöllner et al., 2010).

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Appendix A

Proteins, peptides, genes, and DNA sequences

Table A.1: Proteins, peptides, genes, and DNA sequences used in this work.

Abbreviation	Representation	Source
AdR	adrenodoxin reductase	<i>Homo sapiens</i>
Adx	adrenodoxin	<i>Bos taurus</i>
<i>Amp^R</i>	ampicillin resistance gene, β -lactamase	<i>Escherichia coli</i>
<i>ars1</i>	autosomally replicating sequence	<i>Schizosaccharomyces pombe</i>
COXVI	cytochrome c oxidase subunit VI	<i>Saccharomyces cerevisiae</i>
CYP11A1	cholesterol side-chain cleavage enzyme	<i>Homo sapiens</i>
CYP11B1	steroid 11 β -hydroxylase	<i>Homo sapiens</i>
CYP11B1	aldosterone synthase	<i>Homo sapiens</i>
CYP17	CYP17A1, 17 α -hydroxylase/17,20-lyase	<i>Homo sapiens</i>
CYP21	CYP21A1, steroid 21-hydroxylase	<i>Homo sapiens</i>
Etp1	electron transfer protein	<i>Schizosaccharomyces pombe</i>
his ₆	hexahistidine tag	artificial
<i>LEU2</i>	β -isopropylmalate dehydrogenase	<i>Saccharomyces cerevisiae</i>
<i>nmt1</i>	no message in thiamine	<i>Schizosaccharomyces pombe</i>
ORI	origin of replication	<i>Escherichia coli</i>
<i>PCMV</i>	promoter	Cytomegalovirus
PfuPOL	DNA polymerase I	<i>Pyrococcus furiosus</i>
Pk	14 aa peptide sequence from P and V proteins	Simian Virus 5
<i>Pnmt1</i>	promoter region of <i>nmt1</i>	<i>Schizosaccharomyces pombe</i>
T4 ligase	ligase	enterobacteriophage T4
TaqPOL	DNA polymerase I	<i>Thermus aquaticus</i>
TurboPfu	modified DNA polymerase I	<i>Pyrococcus furiosus</i>
<i>ura4</i>	orotidine 5'-phosphate decarboxylase	<i>Schizosaccharomyces pombe</i>

■

Appendix B

Dragan et al. (2005)

Efficient conversion of 11-deoxycortisol to cortisol (hydrocortisone) by recombinant fission yeast *Schizosaccharomyces pombe*

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Abstract

Genetically engineered microorganisms are being increasingly used for the industrial production of complicated chemical compounds such as steroids; however, there have been few reports on the use of the fission yeast *Schizosaccharomyces pombe* for this purpose. We previously have demonstrated that this yeast is a unique host for recombinant expression of human CYP11B2 (aldosterone synthase), and here we report the functional production of human CYP11B1 (steroid 11 β -hydroxylase) in *S. pombe* using our new integration vector pCAD1. In the human adrenal, the mitochondrial cytochrome P450 enzyme CYP11B1 catalyses the conversion of 11-deoxycortisol to cortisol, a key reaction in cortisol biosynthesis that in addition is of fundamental interest for the technical synthesis of glucocorticoids. We observed that the endogenous mitochondrial electron transport system detected previously by us is capable of supplying this enzyme with the reducing equivalents necessary for steroid hydroxylation activity. Under optimised cultivation conditions the transformed yeasts show in vivo the inducible ability to efficiently and reliably convert deoxycortisol to cortisol at an average rate of 201 $\mu\text{M d}^{-1}$ over a period of 72 h, the highest value published to date for this biotransformation. © 2004 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Cortisol; CYP11B1; Directed integration; Fission yeast; *Schizosaccharomyces pombe*; Steroid 11 β -hydroxylation

1. Introduction

The present state of yeast expression technology offers a great potential for the production of pharmacological target proteins and various enzymes of industrial interest. In the academic field researchers have made use of the fission yeast *Schizosaccharomyces pombe* for decades and it has proven to be a very suc-

cessful model for the investigation of various gene functions like cell cycle studies [1], and after the complete sequencing of its genome [2] and with the wide variety of established genetic methods available [3,4] there is potential for more biotechnological applications of this organism. Previous investigations of our group have suggested that one promising application of recombinant fission yeasts are steroid hydroxylations catalysed by cytochrome P450 enzymes (P450s) [11].

Mammalian cytochrome P450 enzymes generally perform oxidation reactions on their organic substrates while reducing molecular oxygen with electrons delivered from NADPH via electron transfer proteins. In the case of the mitochondrially localised P450s, these two proteins

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are adrenodoxin (Adx) and adrenodoxin reductase (AdR). The second is a flavoprotein which oxidises NADPH and transfers electrons on the iron-sulfur protein Adx, which in turn supplies electrons to the reaction cycle of the P450. A prominent example of a mitochondrial P450 is CYP11B1 (steroid 11 β -hydroxylase), which is expressed in the *zona fasciculata/reticularis* of the human adrenal and converts 11-deoxycortisol (RSS) to cortisol (F), the major human glucocorticoid [6].

Glucocorticoids are used as important antiinflammatory drugs and generally require an 11 β -hydroxy group as a functionally essential entity. During the industrial synthesis of these compounds, the microbiological introduction of the 11 β -hydroxy group into the steroid scaffold represents the most costly synthesis step and also the one wherein most of the losses occur due to the formation of by-products. Several decades ago, studies were done to identify 11 β -hydroxylating microorganisms, and it was shown that the fungi *Cunninghamella blakesleeana* or *Curvularia lunata* could convert RSS, which is accessible from diosgenin or sterols, to cortisol [7,8]. The 11 β -position is axial and therefore more strongly hindered by 1,3-diaxial interactions with the C18- and C19-methyl groups and the 8 β -hydrogen atom than the equatorial 11 α -position. This is considered to be the reason why 11 β -hydroxylation proceeds with lower yields and more side reactions as compared with the 11 α -hydroxylation [9]. Comparative studies on 11 β -hydroxylase activity demonstrated *C. lunata* to be more effective than *Streptomyces fradia* and *C. blakesleeana* [10]. With the tremendous advance of molecular biology since the first cortisol-producing bioconversions were undertaken, expression of CYP11B enzymes in microorganisms emerges as the probable path that development in this field will take. While no functional bacterial expression systems for human or bovine CYP11B enzymes are at hand, efforts have concentrated on using the yeasts *Saccharomyces cerevisiae* [5] and *S. pombe* [11], and cortisol-producing strains have been developed. However, the highest bioconversion activities published to date were in the range of 13 $\mu\text{M d}^{-1}$ [5] and therefore too low to consider their use for industrial applications. The aim of this study was: (i) to create a fission yeast strain that strongly and stably expresses human CYP11B1 and (ii) using this strain to improve cortisol production on the laboratory level to conversion rates that come in the vicinity of industrial relevance.

2. Materials and methods

2.1. Chemicals

Radioactive 11-deoxycortisol was obtained from NEN (Boston, MA), non-radioactive steroids were from Sigma (Deisenhofen, Germany).

2.2. Media and general techniques

Media and genetic methods for studying fission yeast have been described in detail [3,4]. General DNA manipulation methods were performed using standard techniques [12].

2.3. Expression vector and cDNA

We developed an integration vector named pCAD1 (Fig. 1) that is an improved version of the pINT5 vector (P. Wagner, unpublished results) and allows strong expression of the gene of interest under control of the *nmt1* promoter [13]. Using pCAD1, the protein of interest may be expressed with two C-terminal tags, a hexahistidine tag and a Pk tag, respectively; while the addition of polyhistidine tags is widely used for purification with metal-chelating resins and sometimes can also have a stabilising effect on the expressed protein [14], the Pk tag represents an immunological epitope that has been shown to work very well in *S. pombe* [15].

For construction of pCAD1-CYP11B1, we PCR-amplified the CYP11B1 cDNA to introduce *NdeI* and *BamHI* sites. The resulting PCR product was cloned into pcDNA3.1/CT-GFP-TOPO (Invitrogen; Carlsbad, CA) and then subcloned into pCAD1 using *NdeI* and *BamHI* to yield pCAD1-CYP11B1. Sequencing of the

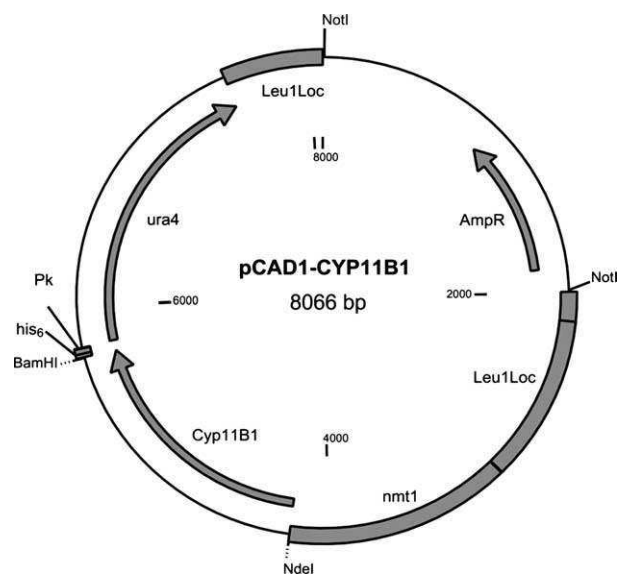


Fig. 1. Vector map of pCAD1-CYP11B1. All sequences are available from the authors by request. Relevant restriction sites are shown. Leu1Loc: gene fragments of the *leu1* gene that serve as integration target sequences; AmpR: ORF for β -lactamase; *nmt1*: *nmt1* promoter; Cyp11B1: ORF for human steroid 11 β -hydroxylase; *his6*: hexahistidine tag; Pk: Pk epitope; *ura4*: ORF for orotidine monophosphate decarboxylase, complements *ura4.d118* in *S. pombe*. A *NotI* digest of this plasmid yields an integration construct with flanking *leu1* sequences.

CYP11B1 cDNA revealed no alterations compared to the wild-type sequence [16].

For transformation of fission yeast, pCAD1-CYP11B1 was digested with *NotI* to yield a 2.0-kb fragment containing the pUC-derived sequences and a 6.1-kb integration fragment containing the expression cassette, the *ura4* marker, and flanking *leu1* sequences. This larger fragment was purified by agarose gel chromatography and used for transformation.

2.4. Transformation of *S. pombe*

We used strain NCYC 2036 (*h⁻ ura4-D18*) for transformation, but any strain containing a functional *leu1* gene and an *ura4* mutant is suited for transformation by pCAD1. Transformation was done using whole cells made competent by the lithium acetate method [4], yielding the new strain SZ1. Transformed cells were plated on EMM with 0.1 g l⁻¹ leucine and 5 μM thiamine and incubated at 30 °C. Transformants were checked for leucine auxotrophy by replica plating, since the targeted integration of pCAD1 by homologous recombination occurs at the *leu1* locus.

2.5. Protein preparation and immunodetection

A total amount of 2.5×10^8 cells were centrifuged at 3000g for 5 min and resuspended in 5 ml water. After a second centrifugation step, the cells were resuspended in 200 μl of 100 mM Tris/SO₄, pH 9.4, mixed with 200 μl of sorbitol and incubated at room temperature for 10 min. Following centrifugation (3000g, 5 min) and discarding of the supernatant, the cells were resuspended in 1 ml of 1.2 M sorbitol, 20 mM KH₂PO₄, pH 7.5, and incubated at 30 °C with 20 mg of Zymolyase 20 T (ICN Biomedicals, Aurora, OH). Incubation at 30 °C was done until the spheroplast ratio reached 80–100%. The cells were then centrifuged (3000g, 5 min) and resuspended in protein extraction buffer (20 mM Tris/Cl, pH 7.5, 5 mM MgCl₂, 2 mM EDTA, 1 mM DTE, 1 g l⁻¹ IGEPAL (Sigma), 1 mM PMSF). Cell breakage was performed in a Potter homogeniser with 900 rpm in an ice water bath.

Nuclei and cell debris were pelleted at 1000g for 5 min; from the supernatant mitochondria were isolated after centrifugation at 10,000g for 1 h and resuspended in 500 μl protein extraction buffer. The supernatant from the mitochondria sedimentation step was considered to be the cytosolic fraction. SDS-PAGE and Western-blot analysis were performed using standard techniques [12]. An α-Pk antibody (MCA1360) obtained from Serotec (Oxford, England) and a secondary peroxidase coupled α-rabbit antibody (DakoCytomation; Glostrup, Denmark) were used for immunologic detection. Visualisation was done using the ECLPlus Western-blot detection kit from Amersham (Piscataway, NJ).

2.6. Steroid hydroxylation assays

Biotransformation of RSS to F was essentially done as described recently [11]. Briefly, SZ1 cells were grown to stationary phase in EMM with leucine, washed once with EMM and resuspended in 10 ml EMM with leucine. RSS was added to a final concentration of 5 mM and 2 μCi [³H]RSS were added for radioactive identification. The assay cultures were shaken in 300-ml Erlenmeyer flasks for 72 h at 30 °C. At several time points samples of 500 μl were extracted with an equal volume of chloroform and separated by HPTLC as described. Steroids were identified after exposure to Fuji imaging plates and quantified on a phosphoimager (BAS-2500, Fuji; Stamford, CT). Daily production rates for cortisol (CR(F)) were calculated from three independent experiments.

3. Results and discussion

3.1. Expression of human CYP11B1 in fission yeast using the integration vector pCAD1

Fission yeast strain NCYC 2036 was transformed using pCAD1-CYP11B1 as described in Section 2. After three days, colonies grown on selective media were tested for the presence of CYP11B1 DNA by PCR. Positive clones were checked for correct integration at the *leu1* locus by replica plating onto agar plates containing phloxine B but lacking leucine. The transformation procedure yielded strain SZ1 (*h⁻ ura4-dl18 leu1::pCAD1-CYP11B1*). For detection of CYP11B1 protein, strain SZ1 was grown in the absence of thiamine to induce the strong *nmt1* promoter, fractionated protein lysates were prepared from this strain as well as from the parental strain NCYC 2036 and examined by Western-blot analysis using an α-Pk antibody. As expected, CYP11B1 could be detected in SZ1 mitochondrial lysates but neither in mitochondrial lysates from NCYC 2036 nor in the cytosolic fraction of SZ1, and its apparent molecular weight of approximately 56 kDa is in good agreement with the calculated mass of 59 kDa (Fig. 2). These data show that, as in the case of human CYP11B2 [11], the mitochondrial localisation sequence of human CYP11B1 is completely functional in *S. pombe*.

3.2. Biotransformation of 11-deoxycortisol to cortisol using strain SZ1

The functionality of the CYP11B1 enzyme was confirmed by steroid hydroxylation assays monitoring the conversion of RSS to F. Mitochondrial P450 steroid hydroxylases like CYP11B1 depend on an electron transport chain that consists of the two proteins adrenodoxin and adrenodoxin reductase. However, we recently

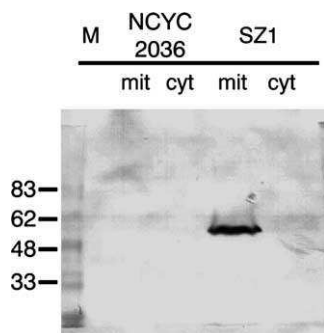


Fig. 2. Detection and sub-cellular localisation of CYP11B1 expressed from pCAD1 in fission yeast by Western-blot analysis. Cytosolic and mitochondrial protein preparations were separated by SDS/PAGE and blotted onto nitrocellulose. Immunological protein detection was carried out using an α -Pk tag antibody as described in Section 2. M: protein standard; SZ1: fission yeast strain expressing the human CYP11B1; NCYC 2036: parental strain of SZ1; cyt: cytosolic fraction; mit: mitochondrial fraction.

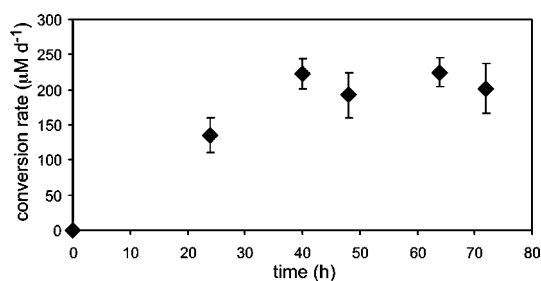


Fig. 3. Time course of the cortisol production activity and the overall daily production of cortisol by SZ1. The assay was carried out as described in Section 2. CR(F) is the overall daily cortisol production rate calculated for the period between $t = 0$ h and the indicated time points. Data shown are mean values of triplicate measurements and standard deviations are shown as error bars.

have reported that after expression of human CYP11B2 (an enzyme that is closely related to CYP11B1) no co-expression of these proteins is needed for efficient substrate conversion by intact fission yeast cells, due to the presence of an endogenous electron transport system [11]. This endogenous electron transport system evidently can also transfer electrons to human CYP11B1, as we could detect high bioconversion activity in strain SZ1 (see below). For improvement of the steroid hydroxylation protocol a number of different assay conditions were tested in order to reach both very efficient and reproducible cortisol production values (data not shown). When RSS bioconversion activity of SZ1 was assayed according to the optimised procedure described

in Section 2, cortisol-producing activity steadily increased from the beginning up to a maximum activity of $225 \pm 20 \mu\text{M d}^{-1}$ measured after 42 h (Fig. 3). For the total assay time of 72 h, bioconversion of RSS to F occurred at an average rate of $201 \pm 36 \mu\text{M d}^{-1}$. During these experiments we discovered two additional steroid bands of varying intensity that result from modifications of the substrate RSS and are apparently not due to CYP11B1 but to endogenous enzymes, as they were also detected in experiments using wild-type fission yeast. The intensity of these activities seems to depend on growth conditions and substrate concentration (data not shown). For calculation of the bioconversion rates presented in this work, these compounds were treated as non-hydroxylated substrate (i.e. RSS) and their formation thus lowered the reported cortisol production rates to some extent. Nevertheless, these activity values are higher by several orders of magnitude than those obtained previously by us using a fission yeast strain that expresses human CYP11B2 (aldosterone synthase) and over-expresses the endogenous ferredoxin *etp1* [11], and they are also significantly higher than the bioconversion rate that was obtained with *S. cerevisiae* expressing bovine adrenodoxin and CYP11B1 [5] (Table 1). It is assumed that this strong increase in biotransformation activity is the result of several causes that act in combination: (1) The CYP11B enzyme. A direct and unambiguous comparison of CYP11B enzyme activities has not been possible so far due to the unavailability of purified human CYP11B1 and CYP11B2. However, after transient expression in mammalian COS-1 cells human CYP11B1 displayed somewhat higher steroid 11 β -hydroxylation activity than either human CYP11B2 [17] or bovine CYP11B1 [18]. (2) The host organism. It is known that the intracellular membrane systems of fission yeast are more highly developed than those of bakers yeast [19], and *S. pombe* has many distinct features in its mitochondria that separate it from other fungi [20]. These facts are reflected by our observations that the localization sequences of human CYP11B enzymes are fully functional in this yeast and that the P450s are supplied with reducing equivalents from endogenous electron transfer proteins like *etp1* [11]. Thus, it can be speculated that the environment of fission yeast mitochondria is more favorable for mammalian mitochondrial proteins than are the mitochondria of other fungi. (3) The biotransformation protocol. Assay conditions that allow a good oxygen saturation of the culture were found to be favorable for efficient sub-

Table 1

Bioconversion of 11-deoxycortisol to cortisol in recombinant yeast strains expressing mammalian CYP11B enzymes

Strain (enzyme, organism)	Assay duration	Conversion rate ($\mu\text{M d}^{-1}$)	Reference
MB224 (human CYP11B2, <i>S. pombe</i>)	24 h	0.0028 ± 0.0003	[11]
TGY73.4/pTG10120 + pTG10350 (bovine CYP11B1, <i>S. cerevisiae</i>)	72 h	12.7 ± 5.2	[5]
SZ1 (human CYP11B1, <i>S. pombe</i>)	72 h	201 ± 36	This work

strate bioconversion (data not shown). However, this point was not investigated systematically.

Our results demonstrate that within a comparably short time significant improvements of the technologically important process under study could be reached, and we are confident that optimisation of yeast strains performing steroid 11 β -hydroxylation by methods of both molecular biology and bioprocess technology has certainly not nearly reached its limits yet for either *S. cerevisiae* or *S. pombe*. For example, experiments aiming at the improvement of the components of mitochondrial P450s by means of molecular evolution have been undertaken (Bichet et al., unpublished results). Moreover, it would be expected that the efficiency of cortisol bioproduction can be further enhanced by scaling-up from shake-flask cultivation to sophisticated high-cell-density fermentation techniques. Thus, it is foreseeable that the current processes of cortisol production which are run with phenotypically highly optimised but not recombinantly modified fungi will encounter serious competition by genetically engineered yeasts in the near future.

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■

Appendix C

Dragan et al. (2006a)

Research Article

Increased TCA cycle activity and reduced oxygen consumption during cytochrome P450-dependent biotransformation in fission yeast

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Abstract

Cytochrome P450s are haem-containing monooxygenases that catalyse a variety of oxidations utilizing a large substrate spectrum and are therefore of interest for biotechnological applications. We expressed human CYP21 in fission yeast *Schizosaccharomyces pombe* as a eukaryotic model for P450-dependent whole-cell biotransformation. The resulting strain displayed strong steroid hydroxylase activity that was accompanied by contrary effects on respiration and non-respiratory oxygen consumption, which combined to a significant decline in total oxygen consumption of the cells. While production of ROS (reactive oxygen species) decreased, the TCA cycle activity increased, as was shown by metabolic flux (METAFoR) analysis. Pentose phosphate pathway (PPP) activity was found to be negligible, regardless of growth phase, CYP21 expression or biocatalytic activity, indicating that NADPH levels in *Sz. pombe* are sufficiently high to support an exogenous P450 without adaptations of central carbon metabolism. We conclude from these data that neither oxygen supply nor NADPH availability are limiting factors in P450-dependent biocatalysis in *Sz. pombe*. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords: biotransformation; cytochrome P450; metabolic flux ratio analysis; oxygen; *Schizosaccharomyces pombe*; steroid hydroxylation

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Introduction

The cytochrome P450s are haem-containing monooxygenases that catalyse a variety of oxidations on a large amount of substrates. P450s utilize two electrons from NAD(P)H to activate dioxygen and are impressive for their ability to catalyse the insertion of oxygen into allylic positions, double bonds, or even into non-activated C–H bonds. This ability to catalyse reactions that are difficult to achieve chemically with high selectivity, especially in water, at room temperature and under atmospheric pressure, makes P450 enzymes attractive for biotechnological applications (Urlacher *et al.*, 2004). However, some major disadvantages still exist, explaining why few industrially relevant processes exist. Most P450s display low

activity, limited stability, dependence on auxiliary electron transfer proteins and need of the expensive co-factors NADH or NADPH. To overcome some of these disadvantages, whole cell oxidations with recombinant microorganisms that express the desired P450s have been developed by many researchers since the respective genes became accessible. In addition, P450s have been the target of extensive protein engineering studies, including sophisticated methods of both rational design and molecular evolution (e.g. Botner *et al.*, 1996; Glieder *et al.*, 2002; Lindberg and Negishi, 1989; Otey *et al.*, 2004, 2006).

Currently, the technical use of P450-dependent biotransformations is restricted to the hydroxylation of steroids and aromatic synthons, and the formation of dicarboxylic acids from alkanes

(Urlacher *et al.*, 2004). However, a large number of reactions were developed at the laboratory scale, using a variety of host organisms (e.g. Dragan *et al.*, 2005; Munzer *et al.*, 2005; Nthangeni *et al.*, 2004), with the total synthesis of hydrocortisone from a simple carbon source by a recombinant *Saccharomyces cerevisiae* strain being a major milestone (Szczebara *et al.*, 2003). The biotechnological prospects of P450-dependent biotransformations are, among other topics (such as enzyme activity and stability), closely related to the questions of co-factor regeneration and oxygen consumption in whole cells, which we address in this study. Human CYP21 (steroid 21-hydroxylase) was expressed in the fission yeast *Schizosaccharomyces pombe* to create a model strain for highly efficient P450-dependent biotransformation in a eukaryote. Using this model strain we investigated whether P450 activity is limited by availability of oxygen or NADPH. We also report the first metabolic-flux and network analysis of fission yeast to provide a reference dataset for *Sz. pombe* and to elucidate whether metabolic fluxes are changed in the process of P450-dependent biotransformation.

Materials and methods

Chemicals

Progesterone, 17 α -hydroxyprogesterone, 11-deoxycorticosterone and 11-deoxycortisol were from Sigma (Deisenhofen, Germany). 16 α -hydroxyprogesterone was from Steraloids Inc. (Newport, RI, USA). Steroid stock solutions were prepared in HPLC-grade ethanol.

Media and general techniques

General DNA manipulation methods were performed using standard techniques (Sambrook *et al.*, 1989). Media and genetic methods for studying fission yeast have been described in detail (Alfa *et al.*, 1993; Moreno *et al.*, 1991). Briefly, strains were generally cultivated at 30 °C in Edinburgh minimal medium (EMM) with supplements of 0.1 g/l final concentration as required. Liquid cultures were kept shaking at 150 r.p.m. The correlation of cell wet weight (CWW) to cell number was calculated according to: $CWW [g] = 3.33 \times 10^{-10} \times \text{cell number}$. Thiamine was used at a concentration of 5 μM throughout. Transformation of fission yeast

strain MB175 (*h⁻ ade6-M210 leu1-32 ura4-d118 his3- Δ 1*) (Burke and Gould, 1994) and immunological protein detections were done as described previously (Dragan *et al.*, 2005).

Expression vector and CYP21 cDNA

We used the commercially available vector pNMT1-TOPO[®] (Invitrogen; Carlsbad, CA), which allows strong expression in fission yeast under the control of the *nmt1* promoter (Maundrell, 1990). Using pNMT1-TOPO[®] the protein of interest is expressed with two C-terminal tags, a hexahistidine tag and a Pk tag, respectively; the Pk tag represents an immunological epitope that has been shown to work very well in *Sz. pombe* (Bureik *et al.*, 2002; Craven *et al.*, 1998). Plasmid pGEM3Z-CYP21 (Nikoshkov *et al.*, 1997), the human CYP21 cDNA, was a kind gift from Walter L. Miller (University of California, San Francisco, CA). From this plasmid the CYP21 cDNA was PCR-amplified using Pfu DNA polymerase (Promega; Madison, WI). After attachment of terminal 3'-adenosines the PCR product was cloned into pNMT1-TOPO[®] to yield pNMT1-hCYP21. Sequencing of the cloned cDNA revealed the following amino acid alterations for the human CYP21 compared to Swiss-Prot entry PO8686: duplication of Leu-9 as in allele CYP21A2*2; exchange Lys102Arg as in allele CYP21A2*3; and a Gly491Val mutation introduced by the reverse primer.

Steroid hydroxylation assays

For steroid bioconversion, cells were grown to early stationary phase (approximately 10^7 cells/ml to 2.5×10^7 cells/ml) in EMM supplemented with adenine, histidine and uracil in the absence of thiamine. After centrifugation and washing with EMM, the cells were resuspended in 10 ml EMM with adenine, histidine and uracil to 5×10^7 cells/ml. The suspension was then transferred to 250 ml Erlenmeyer flasks where steroid concentrations were adjusted to 1 mM. The 10 ml assay cultures were shaken in Erlenmeyer flasks for 72 h at 30 °C and 300 r.p.m. Samples were taken at 0, 24, 48 and 72 h. Steroids were extracted with chloroform and analysed on a Jasco HPLC instrument (Tokyo, Japan) composed of an auto-sampler AS-950, pump

PU-980, gradient mixer LG-980-02 and an UV-detector UV-975 equipped with a reversed phase Nova-Pak[®] C18 column from Waters (Milford, MA, USA). Absorption was recorded at 240 nm, peak detection and quantification were done using the algorithm of the analysis software Borwin[™] version 1.50 from Jasco. Dilutions of respective pure steroids were used as references and as internal standard references as well as for calibrations.

Oxygen consumption rate measurements

The change in the oxygen concentration c_{ox} of a cell suspension solution is given by:

$$\frac{dc_{\text{oxy}}}{dt} = k_L a (c_{\text{sat}} - c_{\text{oxy}}) + r \cdot X \quad (1)$$

where $k_L a$ is the mass transfer coefficient of oxygen between atmosphere and solution, c_{sat} is the oxygen concentration at saturation, r is the respiration rate per biomass and, finally X is the biomass. In this work, the evolution of X with time is negligible because of the short measuring period compared to the generation time of *Sz. pombe*. Fission yeast cells were cultured as described in the section on steroid hydroxylation assay, under fully induced expression conditions, washed once with EMM and resuspended in fresh EMM plus necessary supplements. Prior to measurement, an aliquot of cells was transferred to a 1.5 ml flask at a cell density of 5×10^6 cells/ml and incubated with the appropriate concentration of steroids in a 2.5% (v/v) ethanolic solution of EMM plus supplements for 5 min. Immediately following the incubation step, aliquots of 200 μl were transferred to the wells of a sensor-bearing microtitre plate (Precision Sensing GmbH, Regensburg, Germany) and oxygen consumption was monitored in 1.5 min intervals for 1 h, using the SAFIR fluorescence reader (Tecan; Geneva, Switzerland). The $k_L a$ value was determined experimentally according to the manufacturer's recommendations and was found to be $0.0119 \pm 0.0002/\text{min}$ in EMM. The integrated form of equation 1 was fitted to the cell suspension data by the least squares method, using ORIGIN (OriginLab, Northampton, MA, USA).

Cytometric detection of reactive oxygen species

Cells were grown as described above, washed once with EMM and resuspended to 5×10^7 cells/ml.

Steroid concentrations were set to 1.0 mM with an ethanol content of 2.5% (v/v); 1 ml was transferred to a modified 1.5 ml Eppendorf vial with a 200 μl pipette tip pushed through the cap and then cut in the middle (so-called tip-tube). This simple modification assured a better aeration during the assay period. After 8 h incubation at 30 °C and 1400 r.p.m., cells were subjected to ROS determination and HPLC analysis. For the cytometric detection of ROS, 250 μl cell suspension were incubated for 30 min at 37 °C with 2 μl 250 μM dehydroethidium (DHE) dissolved in DMSO. After washing with water, cells were resuspended in 1 ml water and analysed on a FACSCalibur instrument (BD Biosciences; Franklin Lakes, NJ), using an excitation wavelength of 488 nm and the detector band pass filter set to 585 ± 21 nm (FL2 channel). This setting allows the detection of both ethidium and 2-hydroxyethidium, both of which are formed from DHE by superoxide anion oxidation (Zhao *et al.*, 2005). At least two cytometric measurements were carried out for each cell suspension solution.

¹³C-labelling experiments

All labelling experiments were done in batch cultures assuming pseudo-steady-state conditions during the exponential growth phase (Fischer and Sauer, 2003; Sauer *et al.*, 1999; Wittmann and Heinzle, 2001). ¹³C-labelling of proteinogenic amino acids was achieved by growth on 5 g/l glucose as a mixture of 80% (w/w) unlabelled and 20% (w/w) uniformly labelled [U-¹³C₆]glucose (¹³C, >98%; Isotech; Miamisburg, OH). Cells from an overnight minimal medium culture were washed and used for inoculation, setting up a cell density of 10^5 cells/ml. Where desired, steroid concentrations were set to 1 mM. The cell suspension was incubated at 30 °C and 300 r.p.m. Cell density was monitored microscopically by counting cells with a haemocytometer. ¹³C-labelled biomass aliquots were harvested during the mid-exponential growth phase at 5×10^6 cells/ml and at early stationary phase at 2×10^7 cells/ml. A sample aliquot was subjected to HPLC steroid analysis. The cells were harvested by centrifugation, washed once with sterile water and hydrolysed in 150 μl 6 M HCl at 105 °C for 24 h. The hydrolysate was dried in a heating block at 80 °C under a constant airflow. The free amino acids were

derivatized at 85 °C for 1 h using 15 µl dimethylformamide and 15 µl *N*-(*tert*-butyldimethylsilyl)-*N*-methyl-trifluoroacetamide (Dauner and Sauer, 2000; Wittmann *et al.*, 2002). GC–MS analysis was carried out as reported recently (Fischer and Sauer, 2003), using a previously described biochemical reaction network (Blank and Sauer, 2004).

METAFO_R analysis using amino acids mass isotopomer data

The GC–MS data represent sets of ion clusters, each showing the distribution of mass isotopomers of a given amino acid fragment. For each fragment α , one mass isotopomer distribution vector (*MDV*) was assigned:

$$MDV_{\alpha} = \begin{bmatrix} (m_0) \\ (m_1) \\ (m_2) \\ \dots \\ (m_n) \end{bmatrix} \text{ with } \sum m_i = 1 \quad (2)$$

where m_0 is the fractional abundance of the lowest mass and $m_{i>0}$ are the abundances of molecules with higher masses. To obtain the exclusive mass isotope distribution of the carbon skeleton, corrections for naturally occurring isotopes in the derivatization reagent and the amino acids were performed as described previously (Fischer and Sauer, 2003), followed by calculations of the amino acid (*MDV*_{AA}) and metabolite (*MDV*_M) mass distribution vectors. Metabolic flux ratios were calculated from the *MDV*_M as previously described for *S. cerevisiae* (Blank and Sauer, 2004).

Results

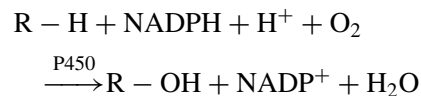
Expression of human CYP21 in fission yeast and steroid bioconversion

Fission yeast strain MB175 was transformed with pNMT1–hCYP21 to yield strain CAD18 (*h-ade6-M210 leu1-32 ura4-D18 his3-Δ1/pNMT1-hCYP21*). For immunological protein detection, cells were grown in the absence of thiamine to induce the strong *nm1* promoter. Protein lysates were prepared from CAD18 and parental strain MB175 and examined by Western blot analysis, using an α -P_k antibody. As expected, the

human CYP21 protein could readily be detected in strain CAD18, but not in the parental strain MB175, and the apparent molecular weight of the enzyme is in good agreement with the calculated mass of 58 kDa (Figure 1A). Cells of strain CAD18 do not show an altered phenotype when observed under the microscope, but grow significantly more slowly than the parental strain (data not shown). Steroid bioconversion assays revealed the strong steroid 21-hydroxylation activity of these cells: The conversion of progesterone to 11-deoxycorticosterone and that of 17 α -hydroxyprogesterone to 11-deoxycortisol were efficiently catalysed by CAD18, with essentially no formation of byproducts under these conditions (Figure 1B). Parental strain MB175 showed no significant conversion of substrates to byproducts except for progesterone, where careful retention time analysis revealed a possible minor contamination of 11-deoxycorticosterone with an unknown product that accounted for less than 4.0% of the total steroid concentration (data not shown). The maximal conversion rate of progesterone is attained during the first 24 h (Figure 1C) and was determined to be $21 \pm 1 \mu\text{M/day}$ [or $1.26 \pm 0.06 \mu\text{mol/g (CWW)/day}$], while for 17 α -hydroxyprogesterone the rate was $259 \pm 9 \mu\text{M/day}$ [or $15.6 \pm 0.5 \mu\text{mol/g (CWW)/d}$], which is one order of magnitude higher than for the former substrate. Biotransformations with recombinant baker's yeast expressing bovine CYP21 reported previously (Sakaki *et al.*, 1990, 1991; Szczebara *et al.*, 2003) might have had significantly lower steroid conversion rates, but the experimental set-up was not entirely comparable. Nevertheless, the strong steroid hydroxylation activity of fission yeast strain CAD18 demonstrates its usefulness as a eukaryotic model for P450-driven biocatalysis. As expected, endogenous P450 oxidoreductase (CPR) is capable of reducing heterologously expressed P450, which renders co-expression of a mammalian CPR unnecessary.

Oxygen consumption and ROS production by fission yeast expressing human CYP21

Cytochrome P450-enzymes generally catalyse hydroxylation reactions of the type:



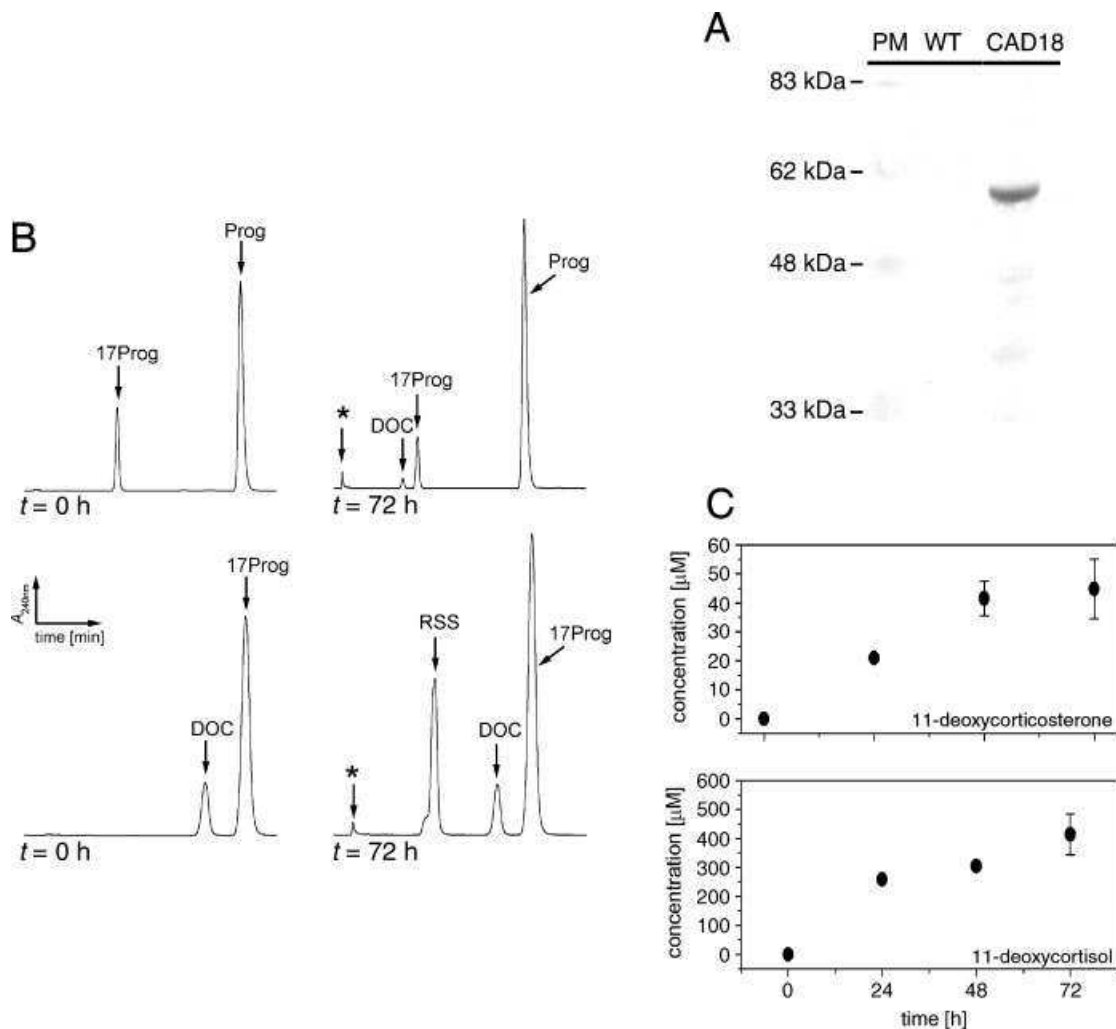


Figure 1. Heterologously expressed human CYP21 can convert progesterone to 11-deoxycorticosterone and 17 α -hydroxyprogesterone to 11-deoxycortisol. (A) Detection of CYP21 protein expression in strain CAD18 by Western blot analysis. After cell lysis, proteins were separated by SDS-PAGE, blotted onto nitrocellulose and probed with α -P₆-tag. Visualization was carried out as described in Materials and methods. WT, wild-type fission yeast strain MB175 (parental strain); PM, protein reference marker. (B) Heterologously expressed human CYP21 is strongly active in fission yeast. The two upper chromatograms (60 min run, 17 α -hydroxyprogesterone as internal standard) show the conversion of progesterone by CAD18, whereas the two chromatograms below (25 min run, 11-deoxycorticosterone as internal standard) show the conversion of 17 α -hydroxyprogesterone by CAD18. The peak denoted by an asterisk elutes at about 2.5 min and appears also in ethanol-treated wild-type fission yeast (data not shown). Prog, progesterone; DOC, 11-deoxycorticosterone; 17Prog, 17 α -hydroxyprogesterone; RSS, 11-deoxycortisol. (C) Time course of product formation. Biotransformation assays were carried out as described in Materials and methods, with 1 mM initial steroid concentrations. At different time points samples were taken from the culture, extracted and analysed by HPLC. Points are mean values with bars representing the standard error of means calculated from at least three independent experiments (non-visible error bars indicate a low standard error)

(Bernhardt, 1996), and the questions of co-factor regeneration and oxygen consumption in whole cells are therefore important issues for the assessment of biotechnological applications. We thus monitored the oxygen consumption

rate of fission yeast with or without CYP21 expression and in the presence or absence of the steroid substrates. Oxygen consumption of the parental strain MB175 is not affected by either steroid as compared to solvent control

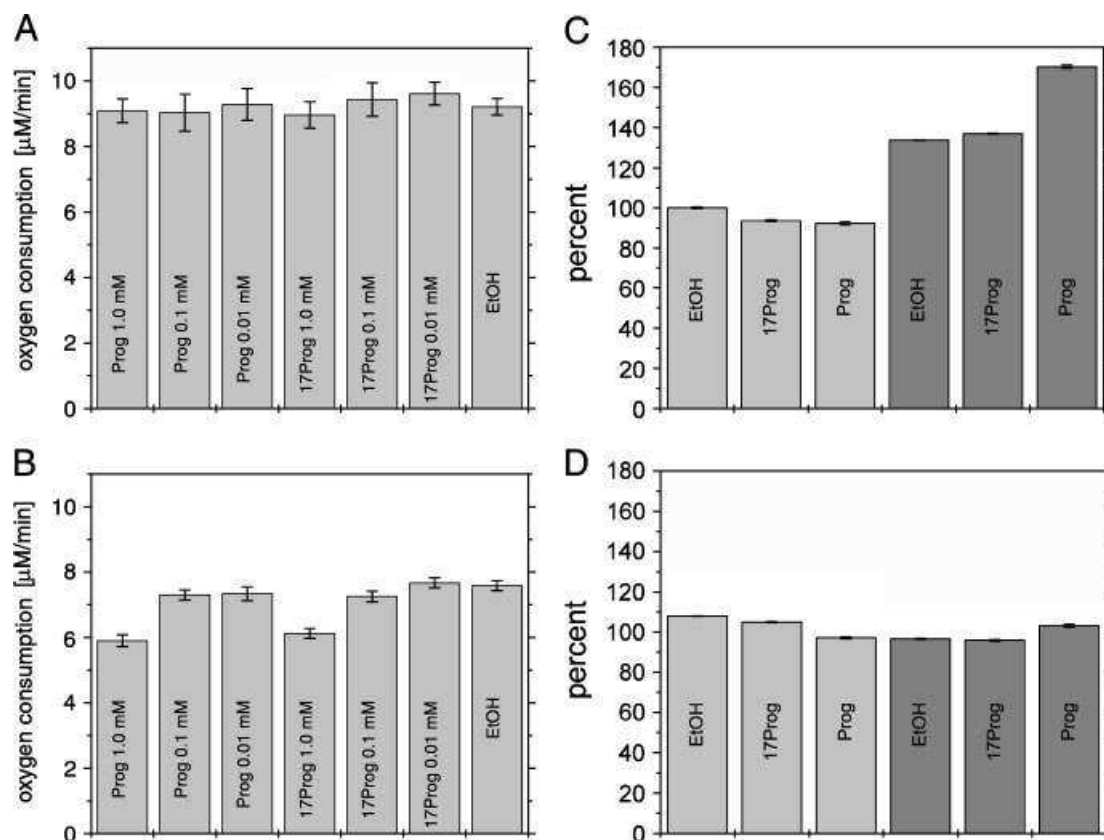


Figure 2. Oxygen consumption and reactive oxygen species production by fission yeast expressing human CYP21. (A, B) Comparison of the oxygen consumption rate of MBI75 (A) and CAD18 (B) in the presence of different concentrations of steroid substrates. Cells were incubated with different steroid concentrations, as indicated, or with 2.5% ethanol (solvent control). Column values represent means gained from eight independent experiments, with standard errors of mean shown as error bars. Prog, progesterone; 17Prog, 17α -hydroxyprogesterone; EtOH, ethanol. (C, D) ROS production by MBI75 (C) or CAD18 (D). Cells were treated as indicated and analysed cytometrically, either directly after addition of steroids or solvent, respectively (light grey bars), or after an incubation period of 8 h (dark grey bars). Fluorescence units were normalized to the solvent control (i.e. ethanol) of the parental strain at $t = 0$ h. Data shown are the means of fluorescence intensity means gained from the FL2 channel, with the standard error of mean calculated using Gaussian error propagation ($n = 6$). EtOH, ethanol; Prog, 1 mM progesterone; 17Prog, 1 mM 17α -hydroxyprogesterone

(Figure 2A). However, we detected a significant decline of oxygen consumption after CYP21 expression in presence of 1.0 mM progesterone or 17α -hydroxyprogesterone, which is not observed at lower steroid concentrations (Figure 2B). More specifically, the oxygen consumption rate dropped from values around $7.6 \pm 0.1 \mu\text{M}/\text{min}$ (solvent control) to $5.9 \pm 0.1 \mu\text{M}/\text{min}$, when cells were treated with 1.0 mM progesterone (two-sided t -test against control; $p = 3.86 \times 10^{-5}$, $n = 8$) and to $6.1 \pm 0.1 \mu\text{M}/\text{min}$ for treatment with 1 mM 17α -hydroxyprogesterone (two-sided t -test against control; $p = 2.67 \times 10^{-5}$, $n = 8$), respectively, which is a relative decrease in oxygen consumption

of about 20% for both substrates. In addition, we generally observed a higher oxygen consumption rate in the parental strain MBI75 (approx. $9.0 \mu\text{M}/\text{min}$) as compared to CAD18 (approx. $7.5 \mu\text{M}/\text{min}$).

At first glance, after the introduction of an additional oxygen-consuming process (i.e. the CYP21 reaction), an increase in oxygen demand of the cells would have been rather expected than the decrease that we observed. In search for an explanation of this observation, we developed an algorithm that simulates non-respiratory oxygen consumption by the heterologously expressed CYP21 and by the endogenous microsomal P450 systems; the latter

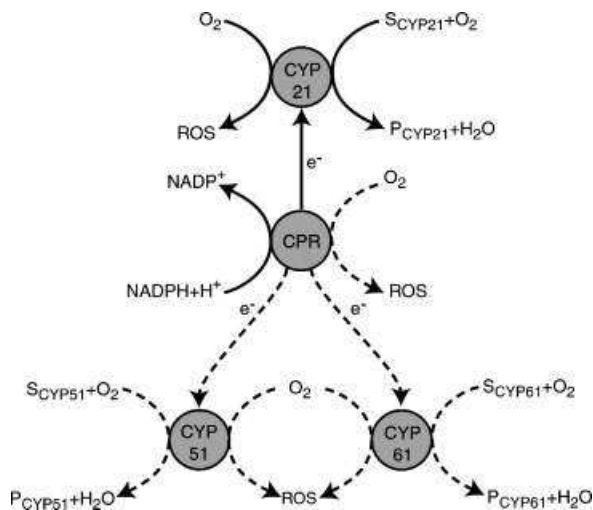


Figure 3. Model of electron flow and oxygen consumption by the microsomal P450 system in fission yeast strain CAD18. CPR is reduced by NADPH and then transfers electrons to either P450s or oxygen. The P450s in turn either hydroxylate their respective substrates (i.e. coupling of NADPH consumption to product formation) or generate ROS (uncoupling). S and P indicate substrates and products for the respective cytochromes. The dashed arrows indicate reactions that are reduced as a result of CYP21 expression

consist of the putative proteins CPR (syn. *ccr1*; a predicted P450 oxidoreductase), CYP51 (syn. *erg11*; a predicted sterol C-14 α demethylase) and CYP61 (syn. *erg5*; a predicted sterol C-22 desaturase), respectively (Wood *et al.*, 2002). Since it is known that the microsomal P450 system of yeast is a major source of reactive oxygen species (ROS; Rosenfeld *et al.*, 2002), the model takes both substrate hydroxylation and ROS production into account (Figure 3). A system of differential equations (described in the Appendix) was used to quantitatively simulate oxygen consumption over time with respect to the amount of CYP21 and its substrates. Of course, these data only describe the oxygen consumption by the cytochrome P450 systems and disregard all other cellular processes that contribute to the overall oxygen consumption of the fission yeast cells. The calculations show that in the presence of the highest concentration of the substrate 17 α -hydroxyprogesterone, oxygen consumption of the system decreases successively with rising CYP21 protein levels (Figure 4A). Likewise, oxygen consumption decreases successively with rising substrate concentrations in the presence of maximum CYP21 concentration (Figure 4B),

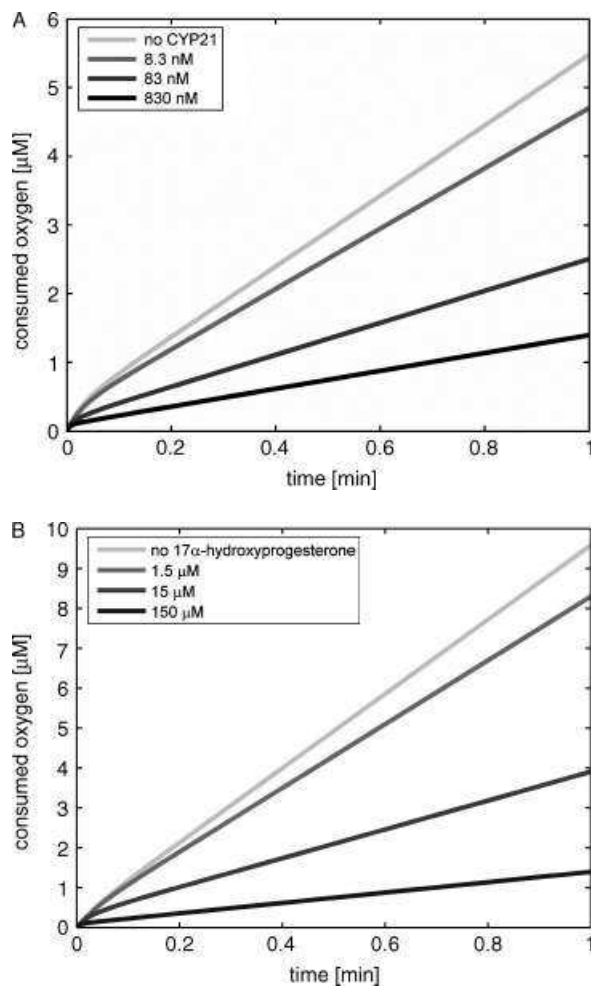


Figure 4. Simulation of total oxygen consumed by the system shown in Figure 1 as a function of time. (A) Simulation of total oxygen consumption against increasing effective concentrations of CYP21 in the presence of the substrate 17 α -hydroxyprogesterone (17Prog). Effective CYP21 concentrations are 0 nM (light grey line), 8.3 nM (middle grey line), 83 nM (dark grey line) and 830 nM (black line), respectively. (B) Simulation of total oxygen consumption against increasing effective concentrations of the substrate 17Prog in the presence of 830 nM CYP21. Effective 17Prog concentrations were 0 nM (light grey line), 1.5 μ M (middle grey line), 15 μ M (dark grey line) and 150 μ M (black line), respectively. For further details on all parameters, see Appendix

as shown for 17 α -hydroxyprogesterone. Similar graphs were obtained using progesterone as substrate (data not shown). The main reason for these effects is the strong complex generation between CYP21 and its substrate (equation A12) followed by efficient formation of the CPR–CYP21–S_{CYP21} complex (equation A18), which counteracts both

CPR-mediated ROS production and interactions between CPR and endogenous CYP51 or CYP61. Importantly, the oxygen concentration in the system is one of the most decisive parameters of the system, with small changes in this value causing significant changes in both oxygen consumption and substrate conversion; by contrast, variation of the NADPH concentration by up to three orders of magnitude does not give rise to strong effects (data not shown).

In order to substantiate the conclusions drawn from the simulation, we cytometrically monitored ROS production in strains CAD18 and MB175 in the presence or absence of steroid substrates (Figure 2C, D). In the parental strain MB175 we observed a slight but significant suppression of ROS production when measured immediately after adding 1.0 mM progesterone (two-sided *t*-test against ethanol value; $p = 9.58 \cdot 10^{-5}$, $n = 6$) or 1.0 mM 17 α -hydroxyprogesterone (two-sided *t*-test against ethanol value, $p = 3.96 \cdot 10^{-4}$, $n = 6$), respectively, to the media (Figure 2C). When treating strain CAD18 with solvent only, a somewhat elevated mean fluorescence value was found as compared to the parental strain, and the same ROS suppressive effect could be observed directly after the addition of steroids (Figure 2D). However, upon longer incubation periods with both steroid substrates, we observed strong differences in the ROS content of both strains. While in MB175 ROS production rises by 40% after prolonged treatment with ethanol or 1.0 mM 17 α -hydroxyprogesterone and even up to 70% after progesterone treatment, this is not at all the case in strain CAD18 (compare dark grey columns in Figure 2C, D). The biotransformation rate of CAD18 was monitored in parallel by HPLC analysis and confirmed strong CYP21 activity (data not shown). These results demonstrate that, even under conditions of strong P450-dependent steroid hydroxylation activity, ROS production is clearly lower in CAD18 than in MB175, which supports our conclusions drawn from the qualitative simulation.

Metabolic flux ratio analysis (METAFor) for *Sz. pombe*

To evaluate the impact of P450-driven biocatalysis on respiratory oxygen consumption and NADPH regeneration, we studied central carbon metabolism of *Sz. pombe* by adapting the metabolic flux

ratio analysis (METAFor) previously used for *S. cerevisiae* and other yeasts (Blank *et al.*, 2005; Blank and Sauer, 2004) to quantify the intracellular carbon flux distribution. When using a new organism for ¹³C-tracer-based flux analysis, the first step is to confirm the assumed network structure (Gombert *et al.*, 2001; Maaheimo *et al.*, 2001). Therefore, we followed our recent report (Blank *et al.*, 2005) to evaluate the compartmented amino acid biosynthesis of *Sz. pombe*. This analysis confirmed that the network structure of *Sz. pombe* is basically identical to the major yeast model *Saccharomyces cerevisiae* (data not shown). To establish the influence of time of harvest for the metabolic flux analysis in *Sz. pombe*, we quantified the intracellular carbon flux distribution during exponential and stationary phase. Interestingly, little change was observed overall, with the non-oxidative pentose phosphate (PP) pathway and the upper bound of the malic enzyme being the notable exceptions (Figure 5A). The very similar datasets might originate from the fact that *Sz. pombe* cannot efficiently utilize ethanol as the sole carbon source (de Jong-Gubbels *et al.*, 1996), so no or little metabolic activity occurs after glucose depletion. In addition, we compared the metabolic flux results to *S. cerevisiae*, a respiro-fermentative yeast (Crabtree-positive), and to *Kluyveromyces lactis*, a respiratory yeast (Crabtree-negative) in Figure 5B. Since *Sz. pombe* is a respiro-fermentative yeast (Heslot *et al.*, 1970; Lloyd *et al.*, 1983), it was not unexpected that its carbon flux distribution was more similar to that of *S. cerevisiae* than to that of *K. lactis*. More specifically, the PP pathway contributes little or not at all to glucose catabolism, little or no malic enzyme or PEP carboxy kinase (gluconeogenesis) activity is measured, and little TCA cycle activity was present under these conditions.

Influence of P450-driven biocatalysis on central carbon metabolism

To investigate the influence of CYP21-driven steroid 21-hydroxylation activity on the flux distribution in central carbon metabolism, we quantified the flux distribution using the above-described ¹³C-based METAFor analysis in strain CAD18. There was essentially no flux through the PP pathway in all experiments with fission yeast cells in this study (Figure 6), regardless of P450 expression and substrate conversion, which suggests a low impact

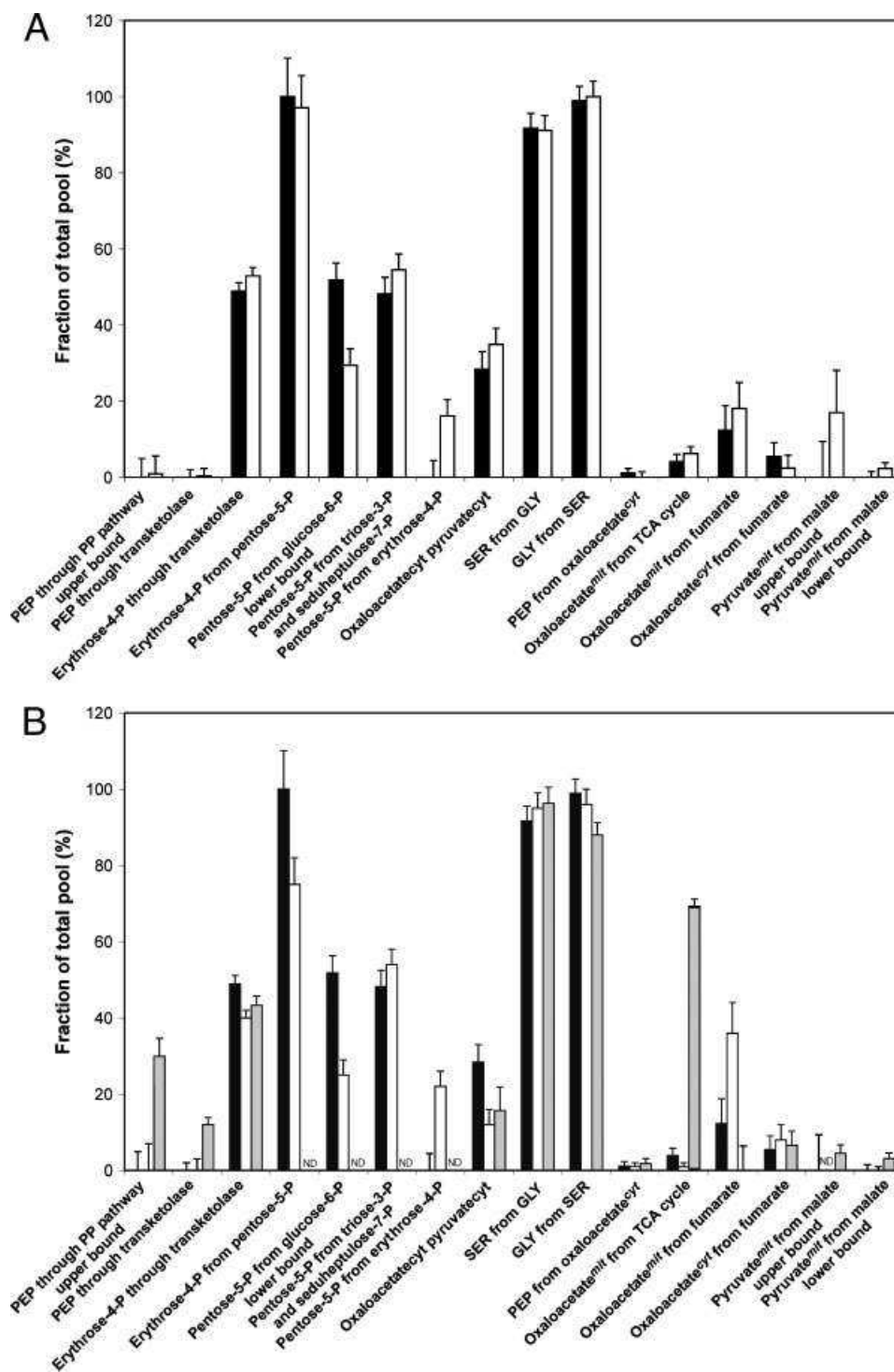


Figure 5. Origin of metabolic intermediates in wild-type fission yeast. (A) Comparison of metabolic fluxes during exponential growth (black bars) and stationary phase (white bars) in glucose batch cultures. (B) Comparison of *Sz. pombe* (black bars) with the respiro-fermentative yeast *S. cerevisiae* (white bars; Blank and Sauer, 2004) and with the respiratory yeast *K. lactis* (grey bars; Blank *et al.*, 2005) during growth in glucose batch cultures. The standard deviations were estimated from redundant mass distributions. *cyt*, cytosolic; *mit*, mitochondrial; ND, not determined

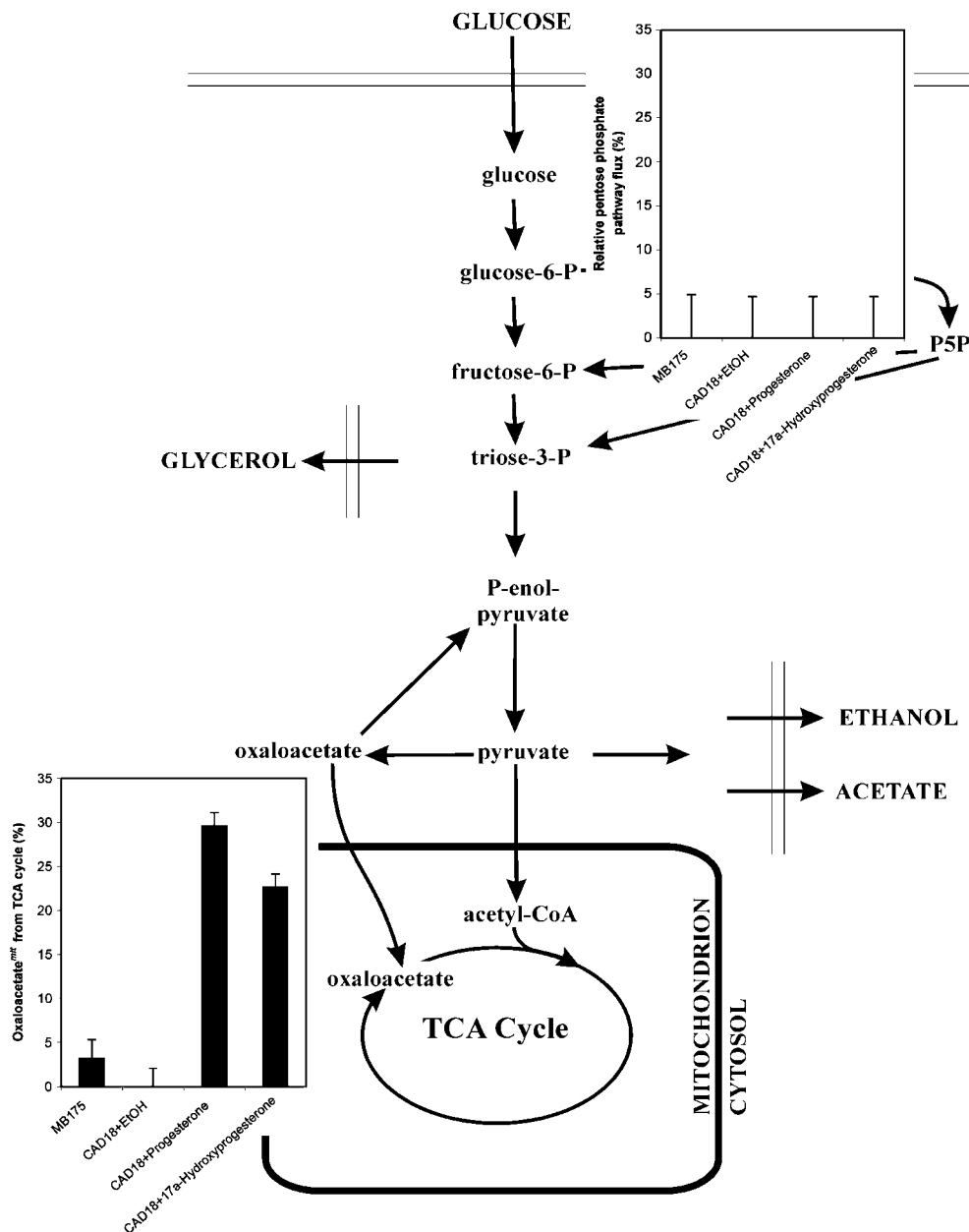


Figure 6. The relative carbon flux distribution at major *Sz. pombe* central metabolism branch points during CYP21-driven biocatalysis. Fission yeast strains and assay conditions are given in the bar graphs. The experimental errors were estimated from redundant mass distributions

of the biocatalytic reaction on the overall NADPH consumption rate. In contrast, the flux through the TCA cycle significantly increased when CYP21 expression was induced and a steroid substrate was present (Figure 6). Thus, P450-dependent steroid bioconversion affects the TCA cycle, but not the PP pathway, in fission yeast.

Discussion

In this study, we created a fission yeast strain (CAD18) that strongly expresses the human steroid hydroxylase CYP21 as a model system for the study of a cytochrome P450-dependent biotransformation in a eukaryotic host. *Sz. pombe* has

previously been used as a host for the expression of microsomal P450s (Yamazaki *et al.*, 1993; Yasumori, 1997, 1999), and the results obtained in those studies as well as this work indicate that the endogenous CPR is capable of reducing heterologously mammalian P450 enzymes. Whole-cell biotransformation experiments using CAD18 demonstrated strong activity of CYP21 expressed in *Sz. pombe*, with steroid hydroxylation rates being significantly higher for the conversion of 17 α -hydroxyprogesterone to 11-deoxycortisol compared to that of progesterone to 11-deoxycorticosterone (Figure 1). This finding correlates well with former reports, where the apparent v_{\max} for 17 α -hydroxyprogesterone was also considerably higher than for progesterone (Lorence *et al.*, 1989; Wu *et al.*, 1991). Unexpectedly, CYP21-expressing cells showed lower oxygen consumption than the parental strain, even in the presence of the steroid substrates (Figure 2).

To explain this finding it can be helpful to remember that CPR supplies reducing equivalents required by P450 isoenzymes (which are a large superfamily of mixed-function oxygenases or monooxygenases found in all three domains of life) and, although there are numerous functional cytochrome P450 genes in mammals (e.g. 57 in human), there is only one CPR gene in each species. Thus, a single CPR is responsible for electron transfer to all the microsomal P450s. The FMN domain of CPR has a similar function to that of the flavodoxins, which contain a single non-covalent-bound FMN prosthetic group, and can substitute for the low-potential ferredoxin during growth under low-iron conditions. The low-potential flavin, FAD, accepts two reducing equivalents from NADPH (dehydrogenase flavin) and the high-potential flavin, FMN, acts as a one-electron carrier (flavodoxin-type flavin) for the net two-electron transfer from NADPH to P450, which in the case of fission yeast are only two: CYP51 and CYP61. Experiments with *S. cerevisiae* suggested that CPR can directly donate electrons to oxygen when P450-dependent activity is low, which accounts for a major part of non-respiratory oxygen consumption (NOC) and causes significant production of ROS (Rosenfeld *et al.*, 2002). After strong overexpression of CYP21 using the *nmt1* promoter (Maundrell, 1990), the amount of electron acceptor proteins available to react with reduced CPR should increase notably, resulting in a drop of CPR-caused

ROS production. CYP21, like all P450s (Yasui *et al.*, 2005), shows a certain degree of uncoupling and therefore, especially in the absence of its steroid substrates, is a source of ROS itself. A qualitative simulation based on a competition model showed that an increase of steroid concentrations should indeed lead to a decline in overall oxygen consumption (Figures 3, 4). This model also explains the difference in basal oxygen consumption between parental strain MB175 and CAD18 even in the absence of CYP21 substrates, and it was corroborated by our finding that ROS production is indeed lower in CAD18 than in MB175 after incubation with progesterone or 17 α -progesterone, respectively (Figure 2). We conclude that this drop in ROS production is the cause for the observed decrease in overall oxygen consumption.

To obtain a reference dataset for the central carbon metabolism of wild-type fission yeast, we performed a metabolic flux ratio analysis (METAFoR) (Blank *et al.*, 2005; Blank and Sauer, 2004; Fischer and Sauer, 2003), which showed few differences between exponentially growing and stationary phase cells and, in addition, confirmed that *Sz. pombe* is a Crabtree-positive yeast (Heslot *et al.*, 1970; Lloyd *et al.*, 1983; Veiga *et al.*, 2000) with a carbon flux distribution that is more similar to that of *S. cerevisiae* than to that of *K. lactis* (Figure 5). More specifically, there is a low flux through the TCA cycle during unlimited growth on glucose, the PPP contributes little or not at all to glucose catabolism, and hardly any malic enzyme and PEP carboxy kinase (gluconeogenesis) activities were measured. After overexpression of CYP21 and in the presence of its steroid substrates, we observed a considerably higher relative TCA cycle flux, while most other flux ratios were rather robust in comparison to the reference (data not shown). The increased TCA cycle flux could reflect a rise in oxygen availability, since ROS production decreases under these conditions (see above).

In *S. cerevisiae*, the PPP and the cytosolic NADP⁺-dependent acetaldehyde dehydrogenase are the main sources of NADPH (Grabowska and Chelstowska, 2003). Fission yeast also displays acetaldehyde dehydrogenase activity (Tsai *et al.*, 1995; de Jong-Gubbels *et al.*, 1996) but it is not known whether this activity is NADP⁺-dependent; the carbon flux through the PPP of *Sz. pombe* remains constantly low, even during

active biotransformation (Figure 6), which suggests that the level of NADPH is sufficiently high in *Sz. pombe* to support an exogenous P450 without adjustment of central carbon metabolism. Yeast promoters are not nearly as strong as their bacterial counterparts, and the membrane-bound eukaryotic P450s display significantly lower turnover rates in comparison to soluble bacterial CYPs, so the problem of co-factor regeneration does not seem to be limiting, at least under these conditions. However, it cannot be excluded that this picture might change when stronger yeast promoters are identified and/or when molecular evolution provides us with P450 mutants that display significantly higher activities.

Acknowledgements

We thank Evi Derouet-Hümbert for help with FACS analysis, Thomas Pleli for help with oxygen measurements and Uwe Sauer for providing access to the GC-MS. This work was supported by a grant of the Bundesministerium für Bildung und Forschung (BMBF) to M. B. (0312641A).

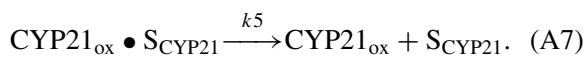
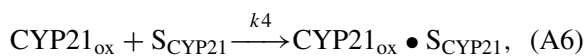
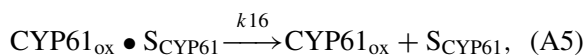
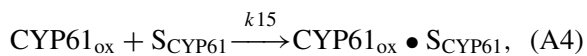
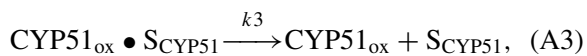
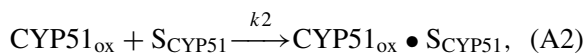
Appendix

Reaction network

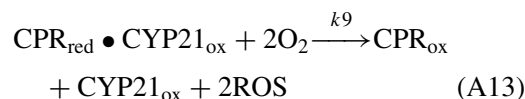
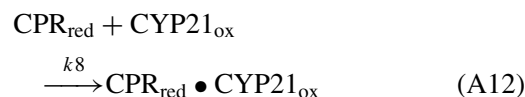
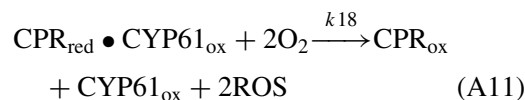
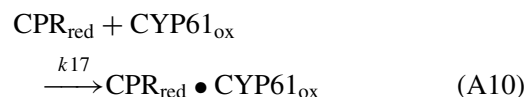
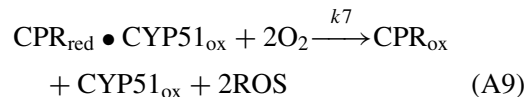
Generation of reactive oxygen species (ROS) by CPR itself (Rosenfeld *et al.*, 2002) is described by:



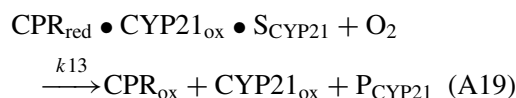
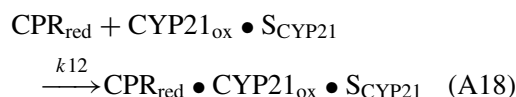
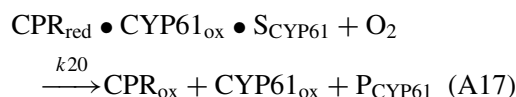
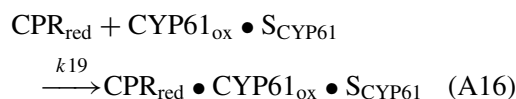
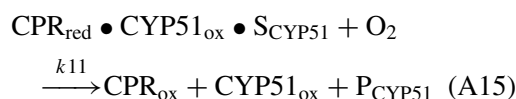
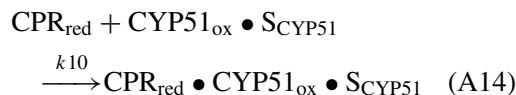
where we simplified the reaction by transferring two electrons on two oxygen molecules. Each of the P450s can form a complex with its substrate (S and P indicate substrates and products for the respective cytochromes) that is subject to a certain degree of decomposition:



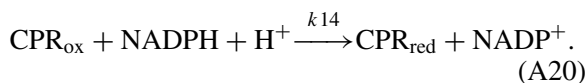
For simplification, the binding of products to the P450s is neglected. We further assumed that the generation of ROS by P450s occurs via an intermediary CPR-P450 complex that, once formed, sequentially binds two molecules of oxygen and produces ROS.



The following reactions describe the formation of products by interaction with CPR:



Finally, the regeneration of reduced CPR is accomplished by:



All molecules are transferred to mathematical symbolism as follows:

y_1 , CPR_{red} ; y_2 , oxygen; y_3 , CPR_{ox} ; y_4 , ROS; y_5 , CYP51_{ox} ; y_6 , S_{CYP51} ; y_7 , complex $\text{CYP51}-\text{S}_{\text{CYP51}}$; y_8 , CYP21_{ox} ; y_9 , S_{CYP21} ; y_{10} , complex $\text{CYP21}-\text{S}_{\text{CYP21}}$; y_{11} , complex $\text{CPR}_{\text{red}}-\text{CYP51}$; y_{12} , $\text{CPR}_{\text{red}}-\text{CYP21}$; y_{13} , $\text{CPR}_{\text{red}}-\text{CYP51}-\text{S}_{\text{CYP51}}$; y_{14} , P_{CYP51} ; y_{15} , complex $\text{CPR}_{\text{red}}-\text{CYP21}-\text{S}_{\text{CYP21}}$; y_{16} , P_{CYP21} ; y_{17} , NADPH; y_{18} , CYP61_{ox} ; y_{19} , S_{CYP61} ; y_{20} , $\text{CYP61}-\text{S}_{\text{CYP61}}$; y_{21} , complex $\text{CPR}_{\text{red}}-\text{CYP61}$; y_{22} , complex $\text{CPR}_{\text{red}}-\text{CYP61}-\text{S}_{\text{CYP61}}$; y_{23} , P_{CYP61} .

The ordinary differential equation system then reads:

$$\begin{aligned} y_1' &= -k_1 y_1 y_2^2 - k_6 y_1 y_5 - k_8 y_1 y_8 \\ &\quad - k_{10} y_1 y_7 - k_{12} y_1 y_{10} + k_{14} y_3 y_{17} \\ &\quad - k_{17} y_1 y_{18} - k_{19} y_1 y_{20} \\ y_2' &= 0 \\ y_3' &= k_1 y_1 y_2^2 + k_7 y_2^2 y_{11} + k_9 y_2^2 y_{12} \\ &\quad + k_{11} y_2 y_{13} + k_{13} y_2 y_{15} - k_{14} y_3 y_{17} \\ &\quad + k_{18} y_2^2 y_{21} + k_{20} y_2 y_{22} \\ y_4' &= k_1 y_1 y_2^2 + k_7 y_2^2 y_{11} + k_9 y_2^2 y_{12} \\ &\quad + k_{18} y_2^2 y_{21} \\ y_5' &= -k_2 y_5 y_6 + k_3 y_7 - k_6 y_1 y_5 \\ &\quad + k_7 y_2^2 y_{11} + k_{11} y_2 y_{13} \\ y_6' &= 0 \\ y_7' &= k_2 y_5 y_6 - k_3 y_7 - k_{10} y_1 y_7 \\ y_8' &= -k_4 y_8 y_9 + k_5 y_{10} - k_8 y_1 y_8 \end{aligned}$$

$$+ k_9 y_2^2 y_{12} + k_{13} y_2 y_{15}$$

$$y_9' = 0$$

$$y_{10}' = k_4 y_8 y_9 - k_5 y_{10} - k_{12} y_1 y_{10}$$

$$y_{11}' = k_6 y_1 y_5 - k_7 y_2^2 y_{11}$$

$$y_{12}' = k_8 y_1 y_8 - k_9 y_2^2 y_{12}$$

$$y_{13}' = k_{10} y_1 y_7 - k_{11} y_2 y_{13}$$

$$y_{14}' = k_{11} y_2 y_{13}$$

$$y_{15}' = k_{12} y_1 y_{10} - k_{13} y_2 y_{15}$$

$$y_{16}' = k_{13} y_2 y_{15}$$

$$y_{17}' = 0$$

$$y_{18}' = -k_{15} y_{18} y_{19} + k_{16} y_{20} - k_{17} y_1 y_{18}$$

$$+ k_{18} y_2^2 y_{21} + k_{20} y_2 y_{22}$$

$$y_{19}' = 0$$

$$y_{20}' = k_{15} y_{18} y_{19} - k_{16} y_{20} - k_{19} y_1 y_{20}$$

$$y_{21}' = k_{17} y_1 y_{18} - k_{18} y_2^2 y_{21}$$

$$y_{22}' = k_{19} y_1 y_{20} - k_{20} y_2 y_{22}$$

$$y_{23}' = k_{20} y_2 y_{22},$$

whereby the prime designates the first derivative with respect to time. Due to the assumption of a steady-state situation, the concentrations of oxygen (y_2), the CYP51 substrate (y_6), the CYP21 substrate (y_9), the CYP61 substrate (y_{19}) and NADPH (y_{17}) were regarded as remaining constant. As indicated in Figure 4, either the concentration of the CYP21 enzyme (y_8) or the concentration of the CYP21 substrate (y_9) was subjected to variation.

Enzyme and substrate concentrations

By deduction from our own unpublished data, the total amount CYP21 enzyme was estimated to be 5 nmol/l of yeast culture. The biotransforming culture was regarded as a two-phase system and, using an average cell volume of $120 \mu\text{m}^3$ (Kubitschek and Ward, 1985), the productive biomass volume of 1 l of culture with a density of 5×10^7 cells/ml was calculated to be 6 ml. This volume is actually harbouring the CYP21; consequently, the effective CYP21 concentration (y_8) is not 5 nM but approximately 830 nM in the biomass phase. Data on the amounts of the relevant endogenous fission

yeast proteins are not available and were arbitrarily set to 83 nM for CYP51_{ox} and CYP61_{ox} (y5 and y18, respectively) and 8.3 nM for CPR_{red} (y1). By deduction from water solubility data for progesterone and 17 α -hydroxyprogesterone (Yang *et al.*, 2002), the effective intracellular concentrations of the CYP21 substrates were assumed to be 40 μ M and 150 μ M, respectively. For the CYP51 substrate lanosterol, data gained with anaerobic fission yeast yielded 0.8 μ g sterol/mg protein and a lanosterol ratio of 4.5% of total sterols. Taking into account the molecular weight of 426.72 g/mol for lanosterol and an average protein content of 10 pg/cell, the lanosterol mole amount per liter culture is 42 nM, which gives roughly 7000 nM (y6) for the biomass phase. It was thus concluded that endogenous CYP51 and CYP61 were virtually saturated with their substrates if we further assume a concentration for the CYP61 substrate ergosta-5,7-dienol (y19), similar to lanosterol. From data reported by Vaseghi *et al.* (1999) and Lloyd *et al.* (1983), the intracellular concentrations of NADPH and oxygen were estimated to be constantly 150 μ M and 500 nM, respectively.

Rate constants

From the data presented in this work, it can be deduced that the formation rates of progesterone and 17 α -hydroxyprogesterone are approximately 1 μ M/h (= 17 nM/min) and 10 μ M/h (= 170 nM/min), respectively, and remain essentially constant during the first 24 h. Together with the values given above, this yields $k_{13} = 0.02$ nM/min for progesterone and $k_{13} = 0.2$ nM/min for 17 α -hydroxyprogesterone, respectively. Using $v_{\max} = 6$ nmol/min/nmol P450 (Kelly *et al.*, 1997) gives $k_{11} = k_{20} = 0.07$ nM/min for both CYP51 and CYP61. The rate constants for substrate binding to the CYP21 were set to $k_4 = 0.0003$ nM/min and $k_5 = 18$ nM/min according to data gained from liposome assays (Kominami *et al.*, 1986). The same substrate kinetics was applied to all constituent P450s in the system. With $v_{\max} = 700$ nmol/min/nmol CPR for either the NADPH oxidation or the cytochrome *c* reduction (Lamb *et al.*, 2001) and 8.3 nM (y1) we used $k_6 = k_{10} = k_{14} = k_{17} = k_{19} = 84$ nM/min. The interaction between fission yeast CPR and human CYP21 (k_8, k_{12}) was assumed to have the same rate constant as between the endogenous P450s and

CPR. For ROS production by CPR (k_1), CYP51 (k_7) and CYP61 (k_{18}), respectively, we assumed a rate that corresponds to 10% of the product rate constant, yielding 0.008/nM²/min for CPR and 0.007/nM²/min for both CYP51 and CYP61.

Summary of all parameters

All the following concentrations are nanomolar and the rate constants are per minute (see respective reaction for first- or second-order dimensions).

Initial concentrations were:

$$\begin{aligned} y_{01} &= 8.3, y_{02} = 500, y_{03} = 0, y_{04} = 0, \\ y_{05} &= 83, y_{06} = 7000, y_{07} = 0, y_{010} = 0, \\ y_{011} &= 0, y_{012} = 0, y_{013} = 0, y_{014} = 0, \\ y_{015} &= 0, y_{016} = 0, y_{017} = 150000, \\ y_{018} &= 83; y_{019} = 7000; y_{020} = 0; \\ y_{021} &= 0; y_{022} = 0 \text{ and } y_{023} = 0. \end{aligned}$$

Rate constants were:

$$\begin{aligned} k_1 &= 0.008, k_2 = 0.0003, k_3 = 18, \\ k_4 &= 0.0003, k_5 = 18, k_6 = 84, k_7 = 0.007, \\ k_8 &= 84, k_9 = 0.007, k_{10} = 84, k_{11} = 0.07, \\ k_{12} &= 84, k_{13} = 0.02 \text{ or } 0.2, k_{14} = 84, \\ k_{15} &= 0.0003, k_{16} = 18, k_{17} = 84, \\ k_{18} &= 0.007, k_{19} = 84, k_{20} = 0.07. \end{aligned}$$

The equation system was numerically integrated using the ode15s solver algorithm (MATLAB; Natick, MA).

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■

Appendix D

Dragan et al. (2006b)

A fission yeast-based test system for the determination of IC₅₀ values of anti-prostate tumor drugs acting on CYP21

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Abstract

Human steroid 21-hydroxylase (CYP21) and steroid 17 α -hydroxylase/17,20-lyase (CYP17) are two closely related cytochrome P450 enzymes involved in the steroidogenesis of glucocorticoids, mineralocorticoids, and sex hormones, respectively. Compounds that inhibit CYP17 activity are of pharmacological interest as they could be used for the treatment of prostate cancer. However, in many cases little is known about a possible co-inhibition of CYP21 activity by CYP17 inhibitors, which would greatly reduce their pharmacological value. We have previously shown that fission yeast strains expressing mammalian cytochrome P450 steroid hydroxylases are suitable systems for whole-cell conversion of steroids and may be used for biotechnological applications or for screening of inhibitors. In this study, we developed a very simple and fast method for the determination of enzyme inhibition using *Schizosaccharomyces pombe* strains that functionally express either human CYP17 or CYP21. Using this system we tested several compounds of different structural classes with known CYP17 inhibitory potency (i.e. Sa 40, YZ5ay, BW33, and ketoconazole) and determined IC₅₀ values that were about one order of magnitude higher in comparison to data previously reported using human testes microsomes. One compound, YZ5ay, was found to be a moderate CYP21 inhibitor with an IC₅₀ value of 15 μ M, which is about eight-fold higher than the value determined for CYP17 inhibition (1.8 μ M) in fission yeast. We conclude that, in principle, co-inhibition of CYP21 by CYP17 inhibitors cannot be ruled out.

Keywords: CYP17, CYP21, fission yeast, prostate cancer, *Schizosaccharomyces pombe*, inhibition, 17 α -hydroxylase/17,20-lyase, steroid 21-hydroxylase

Introduction

Prostate cancer is the second leading cause of death from cancer and the most prevalent cancer amongst men in the western world. Since approximately 80% of human prostatic tumors are androgen dependent, inhibitors of enzymes involved in the key steps of androgen synthesis are of potential pharmacological importance. The cytochrome P450 dependent steroid 17 α -hydroxylase/17,20-lyase (CYP17) is one of these key target enzymes [1,2] as it catalyzes the 17 α -hydroxylation of pregnenolone (Preg) and progesterone (Prog) and the subsequent cleavage of the C20,21-acetyl group to yield dehydroepiandrosterone (DHEA) and androstenedione (AD), respectively [3].

As CYP17 is expressed in the adrenals and testes [4], its inhibition should decrease the production of both testicular and adrenal androgens. Ketoconazole, an antimycotic and unspecific inhibitor of several CYP enzymes that also inhibits CYP17, has been used clinically in the treatment of advanced prostate cancer [5–7]. Although this compound had shown anti-tumor activity, it was withdrawn from clinical use because of its short half-life and its non-selective side effects. Consequently, several research groups have aimed for new steroidal and non-steroidal compounds with CYP17 inhibitory potency [1,8–13]. However, little information has been available so far about a possible co-inhibition of steroid 21-hydroxylase (CYP21) by these compounds. Human CYP21 is a

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Table I. Parental strain and derived strains used in this work.

Strain	Genotype	Expressed P450	Reference
MB175	<i>h- ade6.M210 leu1.32 ura4.dl18 his3.Δ1</i>	none	[24]
CAD8	<i>h- ade6.M210 leu1.32 ura4.dl18 his3.Δ1 / pNMT1-hCYP17</i>	CYP17	this work
CAD18	<i>h- ade6.M210 leu1.32 ura4.dl18 his3.Δ1 / pNMT1-hCYP21</i>	CYP21	[23]

microsomal cytochrome P450 enzyme that is closely related to CYP17 and converts Prog to 11-deoxycorticosterone (DOC) and 17 α -hydroxyprogesterone (17Prog) to 11-deoxycortisol (RSS). The activity of this enzyme is essential for the formation of glucocorticoids and mineralocorticoids, and its impairment causes congenital adrenal hyperplasia (CAH) [14,15], the most frequent inherited disorder of steroid metabolism. In these patients, adrenocorticotrophic hormone (ACTH) levels increase because of defective cortisol synthesis, which results in overproduction and accumulation of cortisol precursors, particularly 17Prog proximal to the block. This in turn causes excessive production of androgens and results in virilization [16]. Human CYP17 and CYP21 differ by only 14 amino acids in length, share 29% amino acid identity, and hydroxylate their steroidal substrates at two carbon atoms that lie a mere 0.26 nm apart. Moreover, the CYP17 and CYP21B genes have identical intron/exon organization [17,18], and are very closely related from an evolutionary point of view [19]. But while the only activities that have been demonstrated for CYP21 are the two 21-hydroxylation reactions mentioned above, CYP17 is not only a 17 α -hydroxylase and a 17,20-lyase, but can also display 16 α -hydroxylase and Δ^{16} -ene synthase activities [20]. Both enzymes share the common substrate Prog, and at least some compounds (e.g. the enantiomer of Prog [21]) competitively inhibit progesterone metabolism of both enzymes. Thus, it cannot *a priori* be ruled out that CYP17 inhibitors significantly inhibit CYP21, which would greatly reduce their pharmacological value. The aim of this study was to develop a rapid and convenient test system that identifies compounds with inhibitory potency towards CYP17 and CYP21. For this purpose we made use of recombinant fission yeast strains that strongly express either human CYP17 or CYP21, respectively, and display high steroid hydroxylation activity. Three CYP17 inhibitors that belong to different structural classes were tested with these strains and the resulting data were compared to the effect of ketoconazole.

Materials and methods

Chemicals

Radioactive [¹⁴C]progesterone was obtained from NEN (Boston, MA), non-radioactive steroids and ketoconazole were from Sigma (Deisenhofen,

Germany). The CYP17 inhibitors Sa 40 [12], YA5ay [11] and BW33 [22] have been described before.

Fission yeast strains and culture

Fission yeast strain CAD18 (all genotypes are listed in Table I) has been described previously [23]. Briefly, it is a derivative of parental strain MB175 [24] and contains the human P450 gene cloned into plasmid pNMT-TOPO[®] (Invitrogen; Carlsbad, CA) that allows strong expression under the control of the *nm1* promoter [25]. Using pNMT-TOPO[®] the proteins of interest are expressed with two C-terminal tags, a hexahistidine tag and a Pk tag, respectively; the latter allows convenient immunological detection of the proteins [26]. Media and genetic methods for studying fission yeast have been described in detail [27,28]. Generally, strains were cultivated at 30°C in Edinburgh Minimal Medium (EMM) with supplements (final concentration 0.1 g·L⁻¹) of adenine, leucine, histidine, and uracil, respectively, as required. Thiamine was used at a concentration of 5 μ M throughout. General DNA manipulation methods were performed using standard techniques [29].

Construction of a fission yeast strain expressing human CYP17

The cDNA of human CYP17 was PCR-amplified using *Pyrococcus furiosus* (Pfu) DNA polymerase (Promega; Madison, WI) and cloned into the fission yeast expression vector pNMT1-TOPO[®] (Invitrogen) to give pNMT1-hCYP17. Fission yeast strain MB175 was the transformed with this plasmid by the lithium acetate method [28] to yield strain CAD8 (all genotypes are listed in Table I). Transformed cells were plated on EMM with 0.1 g·L⁻¹ adenine, histidine, uracil and 5 μ M thiamine and incubated at 30°C. Transformants were checked for plasmid incorporation by colony PCR.

Protein detection

Early stationary phase cultures were used for denaturing protein extraction, where a total amount of approximately 2.5·10⁸ cells were processed as previously described [23]. Protein preparation, SDS-PAGE and Western blot analysis were performed using standard techniques [29]. An α -Pk antibody (MCA1360, Serotec; Oxford, England) and a secondary peroxidase coupled α -rabbit antibody (DakoCytomation; Glostrup, Denmark) were used

for immunologic detection. Visualization was done using 2.0 mL of 2 mg·mL⁻¹ chloronaphthol in 98% EtOH mixed with 25 mL PBS and 10 μL H₂O₂ (30%).

Medium scale steroid hydroxylation assays

For steroid bioconversion, cells were grown to early stationary phase in EMM with supplementes but without thiamine, centrifuged, washed and resuspended in 10 mL of the same media to a cell density of approximately 5·10⁷ cells·mL⁻¹. The cell suspension was then transferred to a 250 mL Erlenmeyer flask, steroid substrate was added to a final concentration of 1.0 mM, and the culture was shaken for 72 h at 30°C and 300 rpm. Samples were taken at 0 h, 24 h, 48 h, and 72 h, respectively. Steroids were extracted with an equal volume of chloroform and analyzed on a HPLC device (Jasco; Tokyo, Japan) composed of an auto-sampler AS-950, pump PU-980, gradient mixer LG-980-02 and an UV-detector UV-975 equipped with a reversed phase Nova-Pak[®] C18 column (Waters; Milford, MA). The mobile phase was methanol:water (60:40) yielding retention times around 45 min for Prog, 20 min for 17Prog, 16.5 min for DOC and 10 min for RSS. Absorption was recorded at 240 nm and peak detection was done using the algorithm of the analysis software Borwin[™] v1.50 (Jasco). Dilutions of respective pure steroids were used as references and as internal standard references as well as for calibrations. In the case of Preg conversion by CAD8, steroids had to be converted to the 4-ene-3-one species by cholesterol oxidase (Serva; Heidelberg, Germany) in order to be detectable at 240 nm. As the activity of cholesterol oxidase is significantly decreased at the low pH values [30] that are typical for fission yeast cultures, Preg conversion was only qualitatively described but not used for quantitative analysis of steroid hydroxylation activity or inhibitor assays.

Screening procedure and determination of IC₅₀ values

Early stationary phase fission yeast cultures were used for miniaturized steroid hydroxylation assays. For each sample, 500 μL of cell suspension was transferred to 1.5 mL Eppendorf tubes and preincubated for 15 min at 30°C and 1400 rpm in a benchtop shaker with different inhibitor concentrations as indicated. After substrate addition, cells were incubated for further a 15 min. Inhibitor concentrations ranged from 100 nM to 20 μM while progesterone concentration was always 100 nM and included [¹⁴C]progesterone for radiocative detection. The reaction was stopped by chloroform extraction of steroids using whole cultures. The organic phase was dried under vacuum, steroids were dissolved in 10 μL of chloroform and spotted on to glass-backed silica-coated HPTLC plates (Kieselgel 60 F₂₅₄, Merck; Darmstadt,

Germany). In addition, small amounts of nonradioactive steroids were spotted as references. The HPTLC was developed twice in chloroform/methanol/water (300:20:1), and steroids were identified after exposure to Fuji imaging plates. Quantification was done using a phosphoimager (BAS-2500, Fuji; Stamford, CT) and the software TINA v2.10 g. The ratio *R* of the respective products was calculated as follows:

$$R_{17\text{Prog}} = \frac{I_{17\text{Prog}}}{I_{17\text{Prog}} + I_{16\text{Prog}} + I_{\text{Bp}} + I_{\text{Prog}}}, \quad (1)$$

$$R_{\text{Doc}} = \frac{I_{\text{DOC}}}{I_{\text{DOC}} + I_{\text{Prog}}}, \quad (2)$$

where *I* is the intensity of the respective steroid as measured by the phosphoimager and the subscript Bp denotes the byproduct (see below). Multiplication of the ratios by the initial concentration of substrate steroid gave the concentration of each steroid at every time point.

Results

Expression of human CYP17 in fission yeast strain CAD8

Fission yeast strain MB175 was transformed using pNMT1-hCYP17 as described above. After three days, the presence of the CYP17 cDNA in colonies grown on selective media was confirmed by colony PCR. The resulting strain was named CAD8. For immunologic detection of the human CYP17 protein, yeasts were grown in the absence of thiamine to induce the strong *nmt1* promoter. Protein lysates were prepared from CAD8 as well as from parental strain MB175 and examined by Western blot analysis using an α-Pk antibody. As expected, the presence of CYP17 could be detected in lysates from CAD8 but not in the parental strain (Figure 1A), and the antibody showed no cross-reaction with other fission yeast proteins.

Steroid bioconversion by strain CAD8

The functionality of the human CYP17 enzyme expressed in *S. pombe* was confirmed by steroid hydroxylation assays monitoring the conversion of Prog and Preg as described above. Both substrates were successfully converted to the respective 17α-hydroxylated products after 72 hours (Figures 1B to 1E). During the first 24 hours, 176 ± 13 μM 17Prog and 83 ± 9 μM 16Prog were produced (Figures 1F and 1G). The concentration-time course is pseudofirst-order for *t* < 48 hours. CYP17 is a microsomal enzyme that in mammalian cells receives electrons from NADPH via the NADPH-cytochrome P450 reductase (CPR), and our results demonstrate that the heterologously

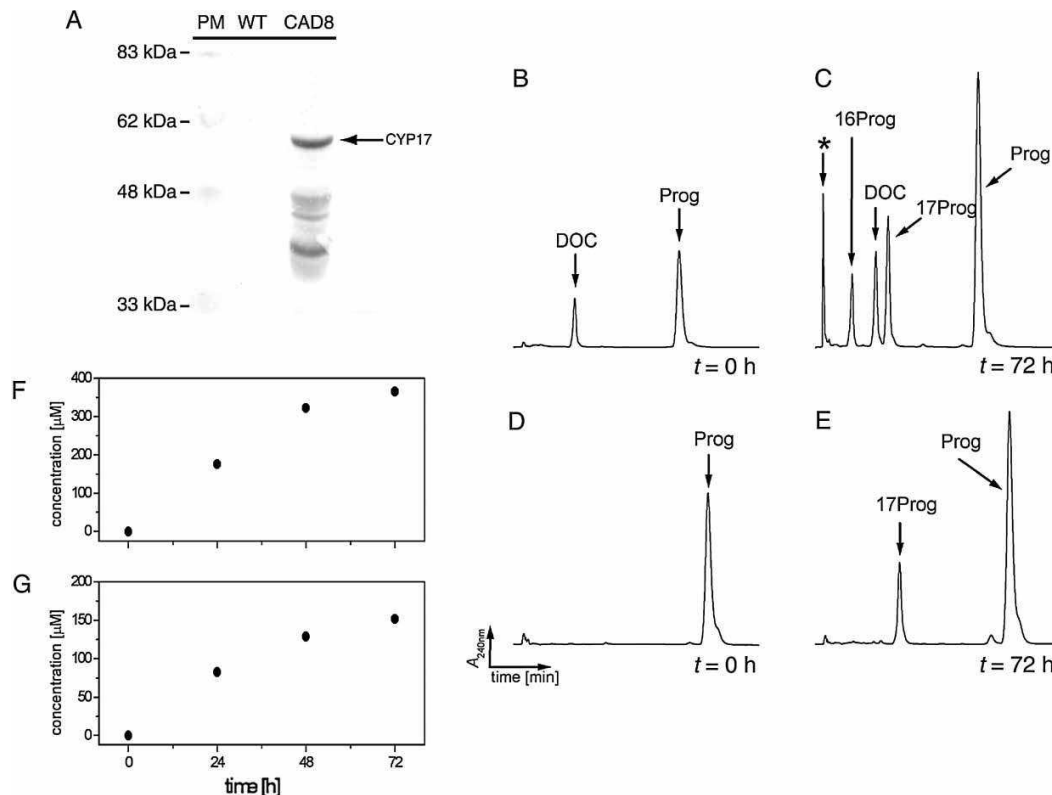


Figure 1. Fission yeast strain CAD8 expressing the human CYP17 can convert progesterone to 17α (hydroxyprogesterone). **A**: Immunologic detection of CYP17 heterologously expressed in strain CAD8. Protein extraction and detection were done as described above. The indicated band is in good agreement with the calculated mass of 59 kDa. PM: protein marker; WT: parental strain (MB175); CAD8: CYP17 expressing strain. **B**, **C**, **D** and **E**: HPLC chromatograms of steroid hydroxylation assays using strain CAD8 as described above. Prog: progesterone, 17Prog: 17α (hydroxyprogesterone), 16Prog: 16α (hydroxyprogesterone), DOC: 11(deoxycorticosterone (internal standard)), *: an unidentified compound that was also detected in the absence of steroid substrates (data not shown). **B** and **C** Conversion of 1.0 mM progesterone by strain CAD8 for 72 h. **D** and **E** Conversion of 1.0 mM pregnenolone by strain CAD8 for 72 h. Prior to steroid extraction with chloroform, the cell suspension was incubated with cholesterol oxidase (see above) in order to convert the Δ_5 -steroids into Δ_4 -species. **F** and **G**: Concentration increase of 17α (hydroxyprogesterone) (**F**) and 16α (hydroxyprogesterone) (**G**) measured during the bioconversion assay with CAD8 incubated with 1.0 mM progesterone. All values were calculated from extraction loss corrected peak areas using calibration with pure steroids and represent mean \pm standard error of mean from three independent experiments.

expressed human enzyme is also efficiently reduced by *ccr1*, the fission yeast CPR homologue [31]. During all experiments, formation of AD or of DHEA (resulting from AD after cholesterol oxidase treatment) was never detected, which indicates that human CYP17 expressed in fission yeast does not catalyze the steroid $17,20$ -lyase reaction under these conditions.

Establishment of a cellular inhibitor assay using fission yeast that expresses either CYP17 or CYP21 and validation of CYP17 inhibition

The functional expression of human CYP21 in *S. pombe* was previously shown by us [23], and the functionality of human CYP17 in this yeast is demonstrated in this study. As a first step towards the set-up of an inhibitor testing procedure, we monitored the conversion of progesterone (Prog) by CYP17 to 17α -hydroxyprogesterone (17Prog) and 16α -hydroxyprogesterone (16Prog), and the CYP21-dependent hydroxylation of Prog to 11-deoxycorticosterone (DOC), respectively, in miniaturized

steroid hydroxylation assays as described above. Under these conditions that employ lower substrate concentrations (100 nM), strain CAD18 (expressing CYP21) converted Prog to DOC virtually to completion within 3 hours without the detectable formation of byproducts (Figure 2). Strain CAD8 (expressing CYP17) converted Prog to 17Prog, 16Prog and a byproduct that appears to be more polar than 16Prog and 17Prog (data not shown). No substrate conversion was observed when using the parental strain MB175 (data not shown). The apparent rate constant of substrate consumption could be determined by fitting the decay function,

$$c(t) = c_0 \cdot \exp(-k_{app}t) \quad (3)$$

with $c(t)$ being the concentration as a time dependent function and k_{app} the apparent substrate consumption rate constant. Data analysis yielded $k_{app} = 2.7 \pm 0.1 \text{ h}^{-1}$ for strain CAD8 (correlation coefficient $r^2 = 0.996$) and $k_{app} = 2.5 \pm 0.1 \text{ h}^{-1}$ for CAD18 ($r^2 = 0.991$).

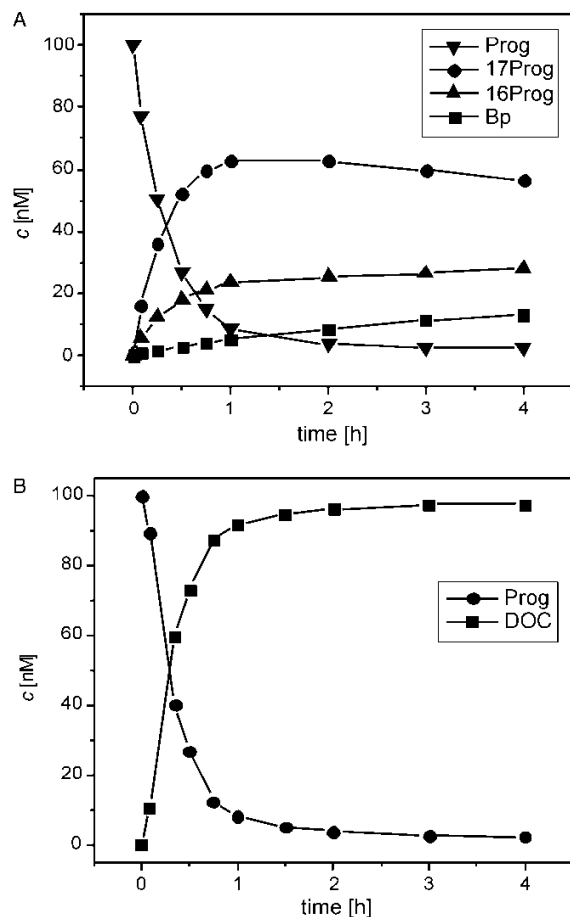


Figure 2. Time course of product formation of fission yeast strains expressing human CYP17 and CYP21. Miniaturized steroid conversion assays were carried out as described in "Materials and methods" with 100 nM progesterone. Samples were taken at the indicated time points, separated by HPTLC and analyzed using a PhosphoImager. Ratios were calculated using Equations (1) and (2) (for the byproducts of the CAD8 assay, Equation (1) was rearranged). Prog: progesterone; DOC: 11-deoxycorticosterone; 17Prog: 17 α -hydroxyprogesterone; 16Prog: 16 α -hydroxy progesterone; Bp: byproduct.

As before, no products of 17,20-lyase activity of CYP17 were detected. Due to these findings, the assay period could conveniently be set to 15 min, where both strains caused between 25 and 40% substrate conversion. Next, we used strain CAD8 to test for the inhibitory action of the broad range P450 inhibitor ketoconazole [32] and of the specific CYP17 inhibitors Sa 40 [12], YZ5ay [11], and BW33 [22] (all structures are shown in Figure 3). Since 17,20-lyase activity was not observed, inhibitory action refers only to the 17 α -/16 α -hydroxylase activity of CYP17. At a concentration of 100 nM, none of the tested compounds displayed a strong inhibition, while at 500 nM, Sa 40 acted as the most potent inhibitor; at 20 μ M, all three specific inhibitors but not ketoconazole strongly reduced CYP17 activity (Table II). IC₅₀ values calculated from these data are presented in Table III. These results indicate that fission yeasts expressing human CYP17 are suitable for rapid inhibitor screening,

although higher inhibitor concentrations were required than in previous assays with human testes microsomes (see Discussion).

Determination of inhibitory action of CYP17 inhibitors on human CYP21

Next, fission yeast strain CAD18 was used for the determination of IC₅₀ values as described in above. An representative experiment is shown in Figure 4, showing the inhibition of CYP21 activity by increasing concentrations of compound YZ5ay. Data calculated from six independent experiments for each compound are shown in Figure 5, and the percent inhibition values for the highest inhibitor concentration used (20 μ M) are given in Table II. In general, all tested compounds inhibited CYP21 less efficiently than CYP17, with YZ5ay being the only compound to cause more than 50% inhibition at a concentration of 20 μ M. Inhibition data points of YZ5ay for the four highest inhibitor concentrations were used to calculate an IC₅₀ of 15 μ M (Table III). Compounds Sa 40 and BW33 were not accessible to reliable IC₅₀ determinations as no inhibition values of more than 50% could be measured. Additionally, we observed an unexpected activation of CAD18-mediated progesterone conversion by ketoconazole of 7% at 2 μ M and 15% at 5 μ M (Figure 5). This behavior reversed into inhibition at concentrations of more than 10 μ M. To eliminate the possibility of a slower diffusion of ketoconazole into the cells and, therefore, of limited access of this compound to CYP21, the time dependence of the product formation ratio on the length of ketoconazole incubation was examined (Figure 6). Cells of strain CAD18 were pre-incubated with 5 μ M ketoconazole for different time periods, while a 60 minute incubation with solvent alone served as control. Prog was then added to all samples, and the steroid conversion assay was performed for 15 minutes as above. This experiment surprisingly showed that CYP21 activity significantly increases with a longer pre-incubation with ketoconazole, which excludes the notion that delayed diffusion of ketoconazole into the cells could account for the weak inhibitory action of this compound in this test system.

Discussion

Heterologous expression of functional human CYP17 in fission yeast

Fission yeast cells strongly expressing human CYP17 did not show an altered microscopic phenotype, grew within one day to early stationary phase under induced conditions (i.e., in the absence of thiamine) and could be directly used for each of the described methods. Western blot analysis of the expressed CYP17 protein revealed a strong band in the expected size range and some additional bands (Figure 1A). Multiple bands in SDS/PAGE were also detected when either

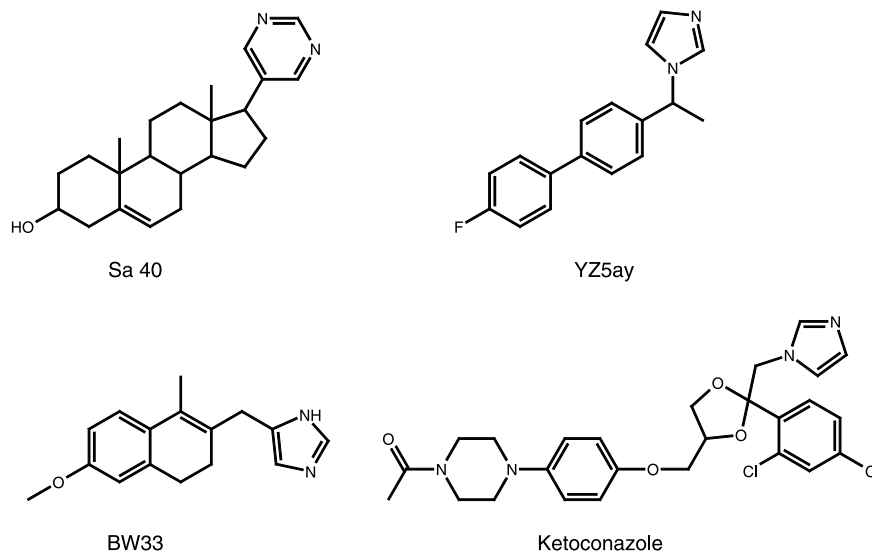


Figure 3. Structures of compounds used in this study.

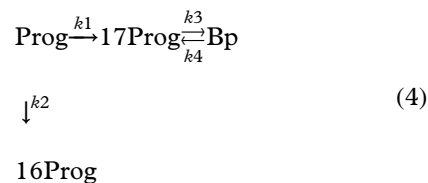
Escherichia coli [33] or *Saccharomyces cerevisiae* [34,35] were used as a host for the expression of human CYP17. Even in the case of COS-1 (African green monkey kidney) cells expressing bovine CYP17, two bands of different intensity were detected [36]. In all studies including this one, the distance between the two highest protein bands indicates a mass difference of roughly 10 kDa, which is by far too large to be accounted for by the loss of the localization signal in the mature protein as compared to the preprotein. Therefore, we assume that overexpressed CYP17 is readily degraded by specific proteolysis, which seems to invariably occur in different hosts including mammalian cells.

The functionality of the human CYP17 enzyme expressed in fission yeast was demonstrated by *in vivo* conversion assays using the natural substrates Preg and Prog (Figure 1). We observed a distinct 17 α - and 16 α -hydroxylase activity of CAD8 towards Preg and Prog, but no detectable 17,20-lyase activity. This suggests that the human P450 can successfully couple to the fission yeast NADPH P450 oxidoreductase (CPR) *ccr1*.

It has been described that the 17,20-lyase activity of human CYP17 is not only dependent on electron delivery from CPR but can be augmented by the presence of cytochrome b₅, even when the latter is not involved in electron transfer itself [34]. It is intriguing that *S. pombe* lacks this function, although its own cytochrome b₅ shares 33% identity and 55% similarity with the human homologue. But *Saccharomyces cerevisiae* strains expressing bovine CYP17 also exhibited poor 17,20-lyase activity towards 17 α -hydroxypregnenolone and nearly none towards 17Prog [37–39]; however, lyase activity was enhanced after co-expression of human cytochrome b₅ [34]. Furthermore, human CYP17 expressed in *E. coli* and

reconstituted with rat CPR showed no detectable lyase activity for 17Prog [33]. Recently, a classification system for CYP17 enzymes from different species was suggested, in which the human and the bovine enzyme are part of the group B CYP17s, which have no or insignificant 17,20-lyase activities in relation to 17Prog [40].

As shown in Figure 1, we detected a byproduct in the 100 nM substrate conversion assays that appeared to be formed only after CYP17 expression (strain CAD8) and not in strains MB175 or CAD18. This strongly points towards a reaction that takes place downstream of the Prog \rightarrow 17Prog/16Prog reaction, whereby the precursor of the byproduct (i.e., either 16Prog or 17Prog) remains to be identified. In order to answer this question by using the time course data obtained in this study, two potential mechanisms can be postulated with rate constants (*k*) indicated above and below the arrows. In Model A, the byproduct (Bp) is made from 17Prog in a reversible reaction:



By contrast, Model B assumes that Bp is made from 16Prog in a reversible reaction:

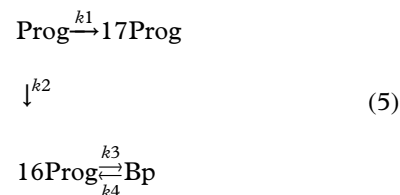


Table II. Percent inhibition of selected CYP17 inhibitors for the 17 α -hydroxylase activity of heterologously expressed human CYP17 (CAD8) and the 21-hydroxylase activity of heterologously expressed human CYP21 (CAD18) in fission yeast at different inhibitor concentrations.

Compound	Percentage inhibition of CYP17	Percentage inhibition of CYP17	Percentage inhibition of CYP17	Percentage inhibition of CYP21
	$c_{inh} = 100$ nM	$c_{inh} = 500$ nM	$c_{inh} = 20$ μ M	$c_{inh} = 20$ μ M
Sa 40	8.0 \pm 2.0	57.0 \pm 3.0	97.3 \pm 0.1	10.0 \pm 1.0
YZ5ay	8.0 \pm 1.0	15.4 \pm 0.4	95.3 \pm 0.1	57.0 \pm 7.0
BW33	7.0 \pm 2.0	0	90.2 \pm 0.2	9.0 \pm 3.0
Ketoconazole	16.0 \pm 2.0	21.9 \pm 0.9	20.7 \pm 2.6	6.0 \pm 3.0

Values represent mean \pm standard error of mean of three independent experiments for the human CYP17 while five different experiments were used for the human CYP21. The concentration of Prog was 100 nM throughout.

Unfortunately the solution set of the dynamic linear equation system is infinite for both models. However, for times $t \leq 0.5$ h we can assume a slow backward reaction of Bp due to relatively low byproduct concentrations bringing about an error of approximately 15% to the solution. Consequently, within the first 30 minutes we further assume the reactions of 17Prog and 16Prog to be described by $c(t) = c_0 (1 - \exp(-k_i t))$, where k_i ($i = 1, 2$) is the apparent substrate consumption rate constant for either 17Prog or 16Prog. Data fitting yielded $k_1 = 1.61 \pm 0.1 \text{ h}^{-1}$ and $k_2 = 0.43 \pm 0.04 \text{ h}^{-1}$. The rate constant for the byproduct reaction was found to be $k_3 = 0.07 \pm 0.01 \text{ h}^{-1}$. The differential equation system was solved numerically using MATLAB's ode45 solver (Natick, Massachusetts, USA) yielding the data presented in Figure 7. Comparison of the simulated versus the experimentally gained data strongly suggests that Bp is made from 17Prog. In case model B would hold, there should be a decrease in 16Prog over time and no significant built up of Bp. Furthermore, there should be no decrease in 17Prog. The decreasing slope of the concentration time course of Bp (Figure 2A) indicates that the back reaction has a rate greater than the Bp production, which was roughly estimated to be about 2 to 4 times higher than k_3 ($k_4 = 3k_3$ in simulation). Remarkably, at low substrate concentrations the steroid conversion rates for CYP17 and CYP21 are roughly equal although there are great differences at 1.0 mM progesterone, where, in agreement with the literature [23], the apparent production rate is ten times lower for DOC than for 17Prog. In baker's yeast expressing CYP17, an endogenous 20 α -hydroxysteroid dehydrogenase (20 α -HSD) was shown to convert 17Prog to 17 $\alpha, 20\alpha$ -dihydroxypregn-4-ene-3-one [39], and *S. pombe* was also reported to exhibit 20 α -HSD activity towards progesterone [41]; in this study, we observed a weak progesterone conversion by wild type fission yeast only at higher substrate concentrations (data not shown). Taken together, it can be assumed that the unidentified byproduct is 17 $\alpha, 20\alpha$ -dihydroxypregn-4-ene-3-one.

IC₅₀ determination with fission yeast expressing human microsomal P450 enzymes

In this study, we report the creation of a fission yeast based test system suited for the determination of IC₅₀ values of inhibitory compounds acting on human CYP17 and CYP21 with an assay duration time of 15 min. The validity of this system was shown by testing three known CYP17 inhibitors with high potency (Sa 40 [12], YZ5ay [11], and BW33 [42]) and ketoconazole [32]. A comparison of IC₅₀ values previously determined in assays using testes microsomal preparations of human CYP17 with data obtained in this study shows that higher inhibitor

Table III. Comparison of IC_{50} values of selected CYP17 inhibitors determined either in human testes microsomes or in fission yeast strains CAD8 or CAD18, respectively.

Compound	IC_{50} CYP17 in microsomes (μM)	IC_{50} CYP17 in fission yeast (μM)	IC_{50} CYP21 in fission yeast (μM)
Sa 40	0.024 [12]	0.8	n.d.
YZ5ay	0.24 [11]	1.8	15
BW33	0.11 [42]	2.8	n.d.

n.d.: Not determined.

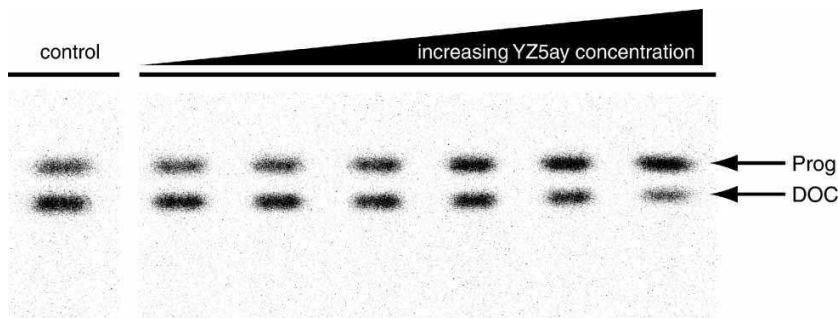


Figure 4. Autoradiographic detection of steroid hydroxylation activity. Cells of strain CAD18 were incubated with increasing concentrations of the CYP17 inhibitor YZ5ay as described in "Materials and methods". Steroids were extracted with chloroform, separated by HPTLC and analyzed using a PhosphoImager. CO: control reaction of strain CAD18 cells (solvent only). Subsequent six lanes contain CAD18 incubations with increasing concentrations of YZ5ay from the left to the right. Prog: progesterone (substrate); DOC: 11-deoxycorticosterone (product)

concentrations are needed in the fission yeast test system to reduce CYP17 activity (Table III). In human testes microsomes, the IC_{50} value of ketoconazole for the inhibition of CYP17 was found to be 740 nM [11], while this compound inhibited CYP21 only weakly [7,43]. This is also reflected by our fission yeast results, where ketoconazole was found to be an ineffective inhibitor of CYP17 or CYP21 even at a concentration of 20 μM (Table II). Of the specific CYP17 inhibitors tested here, only YZ5ay displayed

significant inhibitory potency towards CYP21, while Sa 40 and BW33 showed a strong selectivity towards CYP17. Even in the case of YZ5ay, the selectivity of this compound is about eight-fold higher towards CYP17 than towards CYP21. Still, these findings corroborate our initial apprehension that CYP17 inhibitors may also co-inhibit CYP21 and stress the necessity to test drug candidates for this co-inhibitory effect.

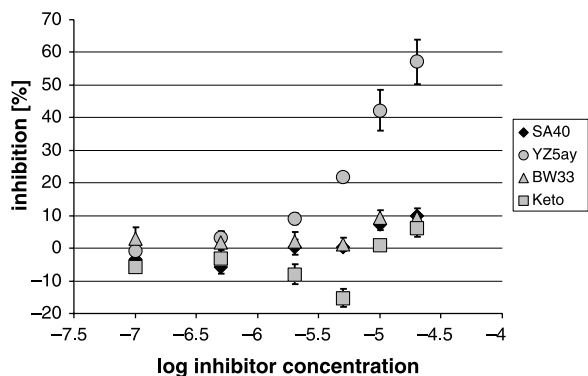


Figure 5. Log inhibitor concentration (CYP21 inhibition plot) for all tested compounds. CAD18 cells were incubated with 100 nM progesterone and increasing concentrations of the different compounds as described in "Materials and methods". Ratios of DOC were normalized to the control reaction and plotted on a half logarithmic scale.

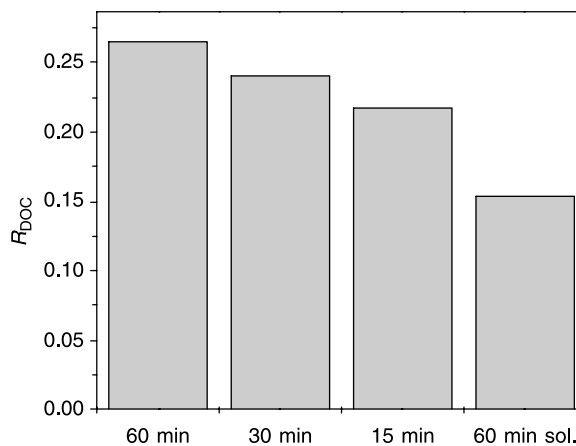


Figure 6. Activating effect of ketoconazole on human CYP21 expressed in fission yeast. Cells of strain CAD18 were incubated with 100 nM progesterone and 5.0 μM ketoconazole for different time periods as indicated. The ratio of DOC at the end of the incubation period was measured as described in "Materials and methods". 60 min sol.: Sample incubated for 60 minutes with solvent only (control).

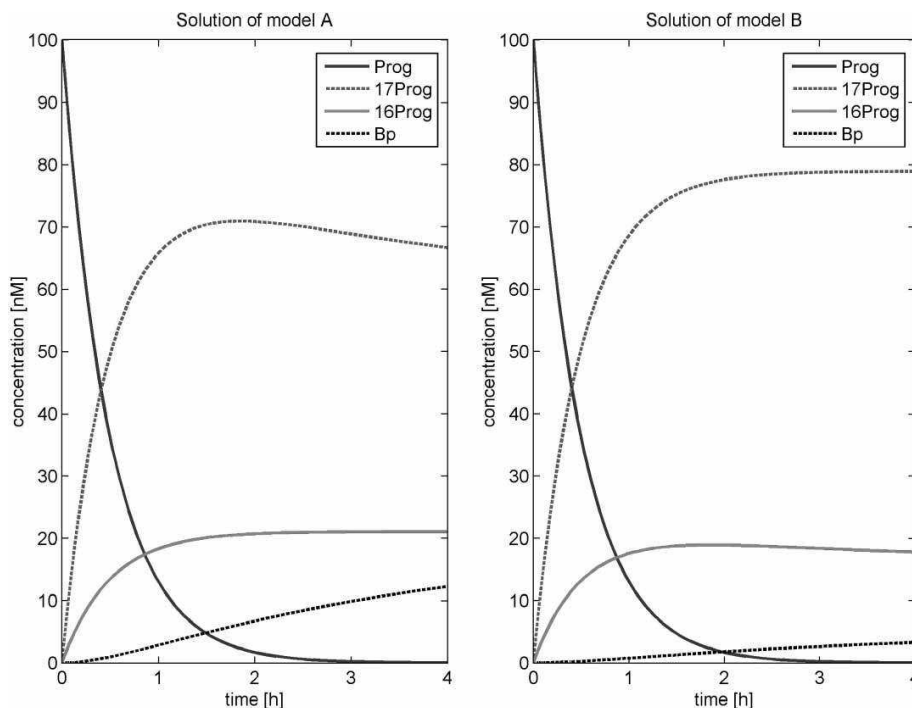


Figure 7. Simulation of the concentration time course in miniaturized progesterone bioconversion assays with fission yeast strain CAD8. The initial progesterone concentration was 100 nM; the rate constants k_1 , k_2 , k_3 and k_4 were set to 1.61, 0.43, 0.07 and 0.21, respectively. The equation systems derived from models A and B (see discussion) were numerically solved using the MATLAB ode45 algorithm under default conditions.

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