

Modulating the Cytochrome P450 system

-

CYP106A2 and various redox partners

Dissertation

zur Erlangung des Grades

des Doktors der Naturwissenschaften

der Naturwissenschaftlich-Technischen Fakultät

der Universität des Saarlandes

von

Tanja Sagadin

Saarbrücken

2026

Tag des Kolloquiums: 18.06.2026

Dekan: Prof. Dr.-Ing. Dirk Bähre

Berichterstatter: Prof. Dr. Rita Bernhardt
Priv.-Doz. Dr. Michael Hutter

Vorsitz: Prof. Dr. Julia Schulze-Hentrich

Akad. Mitglied: Dr. Mark Lommel

Abstract

Cytochromes P450 are ubiquitous enzymes that are able to convert a diverse range of substrates. The role of redox proteins providing the electrons in the P450 system must be carefully sorted out to enable the specific conversion one aims for.

The work overall reports on the different redox systems used specifically with the bacterial CYP106A2 for electron transfer. CYP106A2 is a well-characterized P450 from *Bacillus megaterium* and is usually paired with the bovine redox system of AdR and Adx(4-108). In a first approach, it was shown that the use of other redox systems alters the product pattern in the conversion of progesterone. These results were bioinformatically elucidated and backed up by further experiments. A second approach dealt with the possibilities to mutate Adx(4-108) so that high yields of the main product of the progesterone model conversion could be realized. The topic of the third approach was the fusion of the redox partners YkuN and Fpr. Long, rigid proline linkers outperformed the shorter proline ones and in general the flexible glycine ones and supported the conversion of model substrates.

Finally, initial results on a fused NADPH-regeneration system as well as the promising results of co-immobilizations via Strep- und His-tag were added to demonstrate further possibilities of P450s and their redox partners.

Zusammenfassung

Cytochrome P450 sind allgegenwärtige Enzyme, die eine Vielzahl von Substraten umwandeln können. Die Rolle der Elektronen-bereitstellenden Redoxproteine im P450-System muss sorgfältig recherchiert sein, um die angestrebte Umwandlung zu ermöglichen.

Die Arbeit berichtet über unterschiedliche Redoxsysteme, die insbesondere beim bakteriellen CYP106A2 zur Elektronenübertragung zum Einsatz kommen. CYP106A2 ist ein gut charakterisiertes P450 aus *Bacillus megaterium* und wird üblicherweise mit dem bovinen Redoxsystem von AdR und Adx(4-108) verwendet. Im ersten Ansatz konnte gezeigt werden, dass die Verwendung anderer Redoxsysteme das Produktmuster bei der Umsetzung von Progesteron verändert. Diese Ergebnisse wurden bioinformatisch erklärt und durch weitere Experimente untermauert. Der zweite Ansatz befasste sich mit den Möglichkeiten, Adx(4-108) so zu mutieren, dass hohe Ausbeuten des Hauptprodukts der Progesteron-Modellumwandlung realisiert werden können. Thema des dritten Ansatzes war die Fusion der Redoxpartner YkuN und Fpr. Lange, starre Prolin-Linker übertrafen die kürzeren Prolin-Linker und generell die flexiblen Glycin-Linker und unterstützten die Umwandlung der Modellsubstrate.

Erste Experimente bezüglich der Erzeugung eines fusionierten NADPH-Regenerationssystems sowie vielversprechende Ergebnisse von Co-Immobilisierungen über Strep- und His-Tag wurden hinzugefügt, um weitere Möglichkeiten von P450s und ihren Redoxpartnern aufzuzeigen.

Danksagung

Mein großer Dank gilt meiner Doktormutter Frau Prof. Dr. Rita Bernhardt für die Möglichkeit, das Enzym meiner Diplomarbeit in diesem spannenden Rahmen weiterzubearbeiten. Ich danke dir, Rita, für das Vertrauen, dieses multidisziplinäre Feld zu erforschen, sowie für die wertvolle Gelegenheit, meine Ergebnisse regelmäßig bei unseren Projekttreffen präsentieren zu dürfen.

Ebenso herzlich danke ich Herrn PD Dr. Michael Hutter für die freundliche Übernahme des Zweitgutachtens sowie für die fachliche Unterstützung und das stete Interesse am Fortgang dieser Arbeit.

Mein Dank gebührt zudem meinen Korrektoren Marcus Jochum, Janosch Marx und Simon Janocha für die investierte Zeit und die kritische Durchsicht dieser Dissertation.

Ein besonderer Dank geht an Dr. Frank Hannemann und Jens Neunzig für den stets konstruktiven fachlichen Austausch und den wissenschaftlichen Rat, vor allem für die Stopped-Flow-Messungen. Ebenso danke ich Mohammad Milhim und Natalia Putkaradze für die Mithilfe beim Vervollständigen der Paper.

Des Weiteren bedanke ich mich bei unseren Projektpartnern für die fruchtbaren Diskussionen, die wertvollen Ratschläge und die netten Abende bei unseren Projekttreffen. Explizit sind hier Herr PD Dr. Michael Hutter und Dr. Jan L. Riehm von der Universität des Saarlandes, Frau Prof. Dr. Vlada Urlacher, Dr. Patrick Bakkes und Stefan Biemann von der Universität Düsseldorf sowie Herr Prof. Dr. Roger Gläser, Herr Prof. Dr. Einicke und Kerstin Thiele von der Universität Leipzig zu nennen.

Ein riesiges Dankeschön gebührt der gesamten Arbeitsgruppe der Biochemie für die sehr angenehme (Arbeits-)Atmosphäre. Danke an Birgit Heider-Lips und Antje Eiden-Plach für die stete Hilfsbereitschaft, die Wärme und so manchen wertvollen Plausch. Bei Gabi Schön möchte ich mich für die unverzichtbare Unterstützung in allen bürokratischen Angelegenheiten bedanken. Ein spezieller Dank gilt auch Benjamin Stenger, Simone Brixius-Anderko und Lisa König für die gemeinsame Zeit am Institut und die gegenseitige Motivation. Auch nicht zu vergessen sind hier Flora, Sascha, Maria, Nico und Maël. Danke für eure Freundschaft und die gegenseitige Unterstützung während der gemeinsamen Promotionszeit.

Meinem ehemaligen Vorgesetzten Vincent sowie meinem aktuellen Chef Thomas danke ich für das ehrliche Interesse am Fortgang dieser Arbeit und ganz besonders für die Flexibilität im Job, die es mir ermöglicht hat, die Endphase dieser Dissertation berufsbegleitend zu bewältigen.

Meiner Familie danke ich für das beständige Nachhaken und die unermüdliche Unterstützung, selbst wenn die „Aufschieberei“ mal wieder Überhand nahm. Danke, dass ihr immer an mich geglaubt habt. Ich bin sicher, ihr atmet jetzt genauso erleichtert auf wie ich.

Mein tiefster und ganz persönlicher Dank gilt jedoch Janosch, Jonathan und Leon. Danke für eure Geduld, für das Übernehmen unzähliger Nachmittage, für euer Verständnis für Zeit alleine und dafür, dass ihr mich immer wieder in die Realität zurückgeholt habt. Ihr habt mich konsequent mit dem besten aller Mittel abgelenkt: unserem Alltag.

Scientific contributions

This thesis is based on three original research papers reproduced in chapter 2. The original manuscripts are printed with the kind permission from the other contributing authors as well as the respective journals.

2.1 Tanja Sagadin, Jan L. Riehm, Mohammed Milhim, Michael C. Hutter, Rita Bernhardt „Binding modes of CYP106A2 redox partners determine differences in progesterone hydroxylation product pattern“, *Communications Biology*, (2018) 1:99, DOI: 10.1038/s42003-018-0104-9

T.S. conceived and conducted most of the experiments, analyzed and interpreted the data associated with the experiments, designed the artwork, and is the main author of the manuscript. J.L.R. and M.C.H. conducted and evaluated MD simulation experiments. M.M. contributed to cloning, expression, and evaluation of the mutant. M.C.H. and R.B. designed the experiments and contributed to conceiving experiments, interpretation of data, and writing of the manuscript. All authors reviewed and gave approval to the final version of the manuscript.

2.2 Tanja Sagadin, Jan Riehm, Natalia Putkaradze, Michael C. Hutter, Rita Bernhardt „Novel approach to improve progesterone hydroxylation selectivity by CYP106A2 via rational design of adrenodoxin binding“, *The FEBS Journal* 286 (2019) 1240-1249, doi: 10.1111/febs.14722

T.S. conceived and conducted most of the experiments, analyzed and interpreted the data associated with the experiments, designed the artwork, and is the main author of the manuscript. J.L.R. and M.C.H. conducted and evaluated MD simulation experiments. N.P. conducted the coupling efficiency experiments. M.C.H. and R.B. contributed to conceiving experiments, interpretation of data, and writing of the manuscript. All authors reviewed and gave approval to the final version of the manuscript.

2.3 Patrick J. Bakkes, Jan L. Riehm, Tanja Sagadin, Ansgar Rühlmann, Peter Schubert, Stefan Biemann, Marco Girhard, Michael C. Hutter, Rita Bernhardt, Vlada B. Urlacher „Engineering of versatile redox partner fusions that support monooxygenase activity of functionally diverse cytochrom P450s“, *Scientific Reports*, 7:9570, DOI:10.1038/s41598-017-10075-w (2017)

The author contributed to conversion experiments with different P450s.

Content

Abstract	III
Zusammenfassung	IV
Danksagung	V
Scientific contributions	VI
Content	VII
1 Introduction	9
1.1 Biotechnology in general	9
1.2 Cytochromes P450 in general	10
1.3 P450 structure and catalytic cycle	11
1.4 Biotechnological potential of Cytochromes P450.....	13
1.5 P450 redox partners in general	15
1.6 CYP106A2 from <i>Bacillus megaterium</i> (<i>B. megaterium</i>).....	17
1.7 Ferredoxins, especially adrenodoxin.....	19
1.8 Reductases, especially adrenodoxin reductase	20
1.9 Aim of the Work	21
2 Scientific articles	23
2.1 Sagadin et al. (2018).....	23
2.2 Sagadin et al. (2019).....	33
2.3 Bakkes et al. (2017).....	44
3 Discussion and conclusion	71
3.1 Redox partner choices	71
3.1.1 Modulation of product pattern by the use of different redox partners	71
3.1.2 Modulation of product pattern by site-directed mutagenesis of redox partners.....	74
3.1.3 Consequences for the choice of redox partners.....	76
3.2 Fusion of redox partners.....	77
3.2.1 Fusion of redox partners using DuaLinX	77

3.2.2	Artificial and natural fusions of P450s with their redox partners	81
3.2.3	Creation of a chimera between AdR and alcohol dehydrogenase of <i>Lactobacillus brevis</i>	82
3.2.4	Creation of a chimera between Arh1 and alcohol dehydrogenase of <i>Lactobacillus brevis</i> (Bachelor's thesis)	84
3.3	Use of immobilization as reusable enzyme source in general.....	85
3.3.1	Immobilization of CYP106A2 via Strep-Tag to MCF-material	87
3.4	Conclusion.....	91
4	References	93
5	Abbreviations	111
6	List of figures	113

1 Introduction

1.1 Biotechnology in general

The term biotechnology has gained a lot of fame in its more than 100-year history. Although the use of biotechnology, e.g. in bread baking, beer brewing or fermentation, began much earlier, it was not described with a special term. The expression was introduced by Károly Ereky, a Hungarian scientist, in a publication in 1919 according to which animal organisms produce food as a biotechnological machine (Fári & Kralovánszky, 2006). Starting with this restriction to animals, the concept of biotechnology was further used for broader areas including microorganisms and plants, and is nowadays in use for a large research and economic field that has opened up. Subdivisions developed and colors were added to specify the branches of biotechnology: e.g. white, red, blue, and green biotechnology defined as work in the fields of industry, medicine/pharma, marine/sea, and agriculture. Applying biotechnology, high product quality can be achieved while reducing the waste volume and the use of resources. Furthermore, biotechnological processes are performed in environmentally friendly settings and are more energy efficient than the use of classical chemistry, where environmentally toxic substances are involved, e.g. halogenated compounds and solvents, as well as use of high temperatures and pressures requiring vast amounts of energy (Lin et al., 2017).

The processes used in biotechnology can be adapted and modified by different measures. One of them, protein engineering, is an approach to optimize given enzymes or even whole organisms in order to increase their output for their (industrial) use. Depending on the state of knowledge (e.g. structure availability), protein engineering can be achieved by directed evolution (Cherry & Fidantsef, 2003; A. Kumar & Singh, 2013) or rational design (Prather & Martin, 2008; Sadeghi et al., 2001). Protein crystallography and computational methods enabled dramatic progress so that protein engineering has been greatly facilitated over the years (McRee & David, 1999; Schwede & Peitsch, 2008).

Regarding the research topic of this Thesis, i.e. research and engineering of enzyme systems like Cytochromes P450 (P450s, CYPs) as well as the progress in application of P450s that has already been established, would also have been unthinkable without biotechnology (C. S. Miles et al., 2000).

1.2 Cytochromes P450 in general

Cytochrome P450s (P450s) are heme proteins and function as external mono-oxygenases. They are present in every area of life, in procaryotes as well as eucaryotes and even in viruses (Lamb et al., 2019) – but not every organism possesses P450s, with the model organism *E. coli* as a well known exception (Kelly & Kelly, 2013). Their first discovery was published in 1958 by Garfinkel as well as by Klingenberg in 1958, both independently describing the properties of carbon monoxide (CO)-binding pigment isolated from pig or rat liver microsomes, respectively (Garfinkel, 1958; Klingenberg, 1958). It was followed by the work of Omura and Sato proving that the causing pigment was actually a protoheme containing protein. They named it „P-450“, because of the unusual CO difference spectrum of the reduced protein exhibiting a peak at 450 nm (Omura & Sato, 1964). As of 2018, the P450 database lists more than 41,000 P450 sequences with assigned names of overall more than 300,000 P450 sequences already mined and collected in preparation for naming (Nelson, 2009, 2018). The nomenclature of cytochrome P450 is quite uncommon for proteins as it does not describe its function but the characteristics of P450s: cytochrome describes the hemoprotein, P the "Pigment", and the number 450 the characteristic peak at 450 nm in case the protein is bound to CO.

P450s perform various reactions that are part of the organism's endogenous metabolism, like steroidogenesis in mammals (Hanukoglu, 1992), or the secondary metabolism in bacteria (Greule et al., 2018). Additionally, they are part of the metabolism of exogenous materials, such as the conversion of drugs in human metabolism (Anzenbacher & Anzenbacherová, 2001), or the conversion of xenobiotics like RDX (Rylott et al., 2006).

Because of the vast number of P450s (Nelson, 2018) found in diverse forms of life, the nomenclature has been standardized regarding their sequence identity (Nebert et al., 1989). P450s with an amino acid sequence identity of more than 40 % belong to one family, whereas an identity of more than 55 % assigns the member to a subfamily. The designation of cytochromes P450 always starts with the abbreviation P450, showing its membership to the cytochrome P450 superfamily, which is followed by an Arabic number depending on the assigned family, a letter depending on the assigned subfamily and another Arabic number for the individual gene. This naming convention results for instance in CYP106A2, identifying one member of the family 106 and the subfamily A.

The function of P450s is the activation of molecular oxygen to introduce one atom of oxygen into e.g. a non-activated C-H bond and reduce the second oxygen atom to water (Figure 1).



Figure 1: General reaction of cytochrome P450 systems

The most common reaction catalyzed is the hydroxylation, but the reaction spectrum which can be accomplished by P450s also covers alcohol oxidation, aldehyde oxidation, sulfoxidation, N-, O-, S-dealkylation, and C-C-bond cleavage (Bernhardt & Urlacher, 2014; Guengerich, 2018; Meunier et al., 2004; Sono et al., 1996). This potential for diverse reactions also influences the fact that P450s can use a broad range of endogenous substrates, such as fatty acids (Hammerer et al., 2018), and steroids, as well as xenobiotics like pharmaceuticals, pesticides, or carcinogens (Bernhardt, 2006).

1.3 P450 structure and catalytic cycle

Despite their diversity in function, substrates, origin species, and their amino acid sequences often being less than 20 % identical, the overall folding structure of P450s is highly conserved. This was further elucidated by the unravelling of more and more crystal structures (Hasemann et al., 1995; Poulos, 1995; Spradlin et al., 2016; Sugimoto et al., 2008; Yano et al., 2000). As increasing structural data became available, the flexibility for substrate entry and subsequent closing of the active site became clear by comparing the substrate-free and substrate-bound forms (Cojocaru et al., 2007; Li & Poulos, 1997). The so-called "induced fit" was observed by Yano (Yano et al., 2000), where it was shown that differently shaped substrates lead to a differently shaped active site. These two facts, the open/closed form as well as the induced fit, are the basis for the range of different accepted substrates for P450s.

The core structure of all P450s consists of a four-helix bundle, which coordinates the prosthetic heme group (Pochapsky et al., 2010; Poulos, 2003, 2005). The heme group is further bound to an absolutely conserved cysteine, which represents the fifth, proximal ligand and is the reason for the formation of the Soret band at 450 nm. The structural diversity of P450s allows them to overall accept many substrates of different sizes via binding at the six flexible "substrate recognition sites" (SRS) (Gotoh, 1992; Peterson & Graham, 1998). The basic catalytic reaction of all P450s follows the same principle of changes of the heme-iron between the two oxidation states (Fe III (ferric) and Fe II (ferrous)) (Denisov et al., 2005).

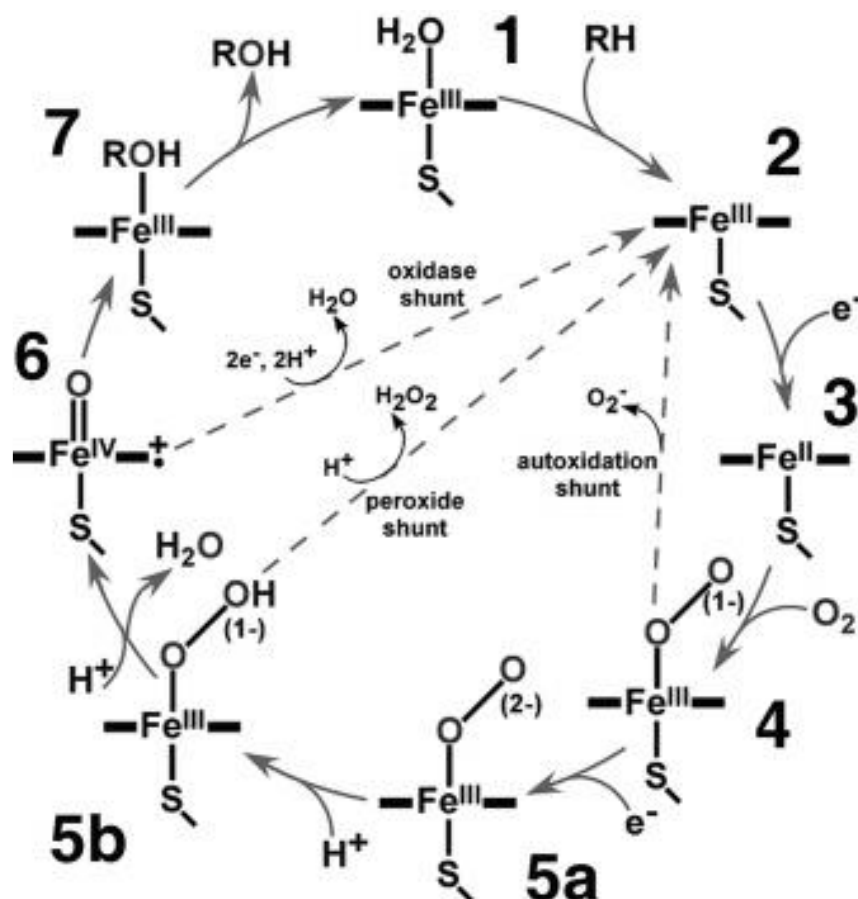


Figure 2: Multi-step catalytic cycle of P450s and shunt pathway (Denisov et al., 2005)

The catalytic cycle of the monooxygenase reaction of a P450 proceeds through eight intermediate steps (see Figure 2). The cycle starts with the heme iron in an inactive state (low spin Fe III), six-coordinated by the four N-atoms of the heme ring (equatorial), the S-atom from cysteine and an axial water molecule (1). Binding of the substrate (RH) displaces the water molecule and forms a five-coordinated high-spin Fe III (2). As one electron is delivered by the redox partner, the Fe III is reduced to Fe II (3). The following binding of molecular oxygen leads to the Fe II-dioxy species (4). The second electron reduces the oxygen and forms the peroxo-Fe III intermediate (5a), which is then protonated to a hydroperoxy-Fe III intermediate (5b). The second protonation leads to the heterolytic cleavage, yielding one water atom and the so-called "Compound I", an iron-oxo ferryl species (6). "Compound I" oxygenates the substrate to form a product complex ("Compound II") and a substrate radical (7). In the last step, the substrate (R-OH) dissociates and the enzyme reverts back to its initial water-bound state in the low-spin Fe III form (1).

During this cycle, reactive oxygen species (ROS) can be produced, and may lead to an inactivation of the enzyme. Furthermore, there are three shunt pathways able to cut short the mechanism before a product is formed, to convert the enzyme back to its resting state (2). These

pathways are called the autooxidation shunt, peroxide shunt, and oxidase shunt and are often referred to as uncoupling (Denisov et al., 2005).

1.4 Biotechnological potential of Cytochromes P450

With the present setup, the product output of most P450 reactions does not yet meet the needed level to match most industrial processes yet (e.g. certain minimal fermenter sizes, or weighable product output). For this reason, the optimization of P450 reactions is well-needed and in the focus of interest to many research groups.

As P450s are complex proteins associated with additional redox partners, their use in industrial processes requires several steps of adaptation beforehand. Until now, the industrial use of P450s is still limited as they exhibit low activity, lack of stability, dependence on cofactors, oxygen, and redox partners, as well as the solubility of substrates (Li et al., 2020; O'Reilly et al., 2011). Regardless of these obstacles, P450s represent excellent potential candidates for the use in industrial approaches (Bernhardt, 2006; Bernhardt & Urlacher, 2014; Grogan, 2011). They have the ability to stereo-selectively introduce oxygen into an inactivated hydrocarbon-bond and are thus able to late-stage functionalize the bioactive substrate for new drug candidates (Di Nardo & Gilardi, 2020). The hydroxylation position is a point of action for further modifications of the origin skeletal structure of complicated molecules, as it facilitates the bioavailability, bioactivity, or solubility (Schmitz et al., 2018). Using classical chemistry, these reactions are hardly realizable. The exorbitant regio- and stereoselectivity of P450s continues to thrive research in this field, leading to highly efficient reactions without the need of expensive product purification processes. A first approach to the engineering of P450 systems represents protein engineering of P450s (Kille et al., 2011), either based on P450's crystal structure or, if this is not elucidated yet, on common P450-structure knowledge via homology models. Possible points of mutations are e.g. substrate binding sites, substrate entry channels, heme coordination residues, or redox partner binding sites (i.e. surface residues). The last point could also be tackled by engineering the involved redox partners (Chun et al., 2007; Ruijsenaars et al., 2007), where the mutation sites might hit at the P450-binding site, but could also influence the redox potential, and therefore the overall conversion. A second approach might be engineering the electron source (Wichmann & Vasic-Racki, 2005): For example by using heterologous enzymes for a coupled cofactor regeneration, e.g. alcohol dehydrogenase (Janocha & Bernhardt, 2013) or α -hydroxyacid oxidase (Gandomkar et al., 2018), or use of light-activated

systems (Gnanasekaran et al., 2016; Nielsen et al., 2013). Furthermore, substrate engineering, such as linking an anchor to the substrate to induce binding of the engineered substrate, can enable or even increase conversions with P450s (Lewis et al., 2009; Sherman et al., 2006). Even the use of decoys (dummy molecules) has been described to modify the active site of the P450, resulting in an altered substrate specificity and regioselectivity of the enzyme (Polic & Auclair, 2014). A fourth approach is metabolic engineering of whole-cell catalysis (Karasawa et al., 2018), by introducing metabolic pathways including P450s in recombinant *Saccharomyces cerevisiae* (*S. cerevisiae*) strains (Szczebara et al., 2003), or by P450-based systematic optimization (Zhao et al., 2016; Zilly et al., 2011).

To date, several P450s have already been further developed to achieve industrial production. The best example was developed by Ro et al.: They described that *S. cerevisiae* is able to produce artemisinic acid, a precursor of the anti-malaria drug artemisinin, using the CYP71AV1 from *Artemisia annua* in high amounts up to 100 mg/L (Ro et al., 2006). Additionally, there have been contributions of P450 research to the field of bioremediation, as one important task of P450s is drug metabolism in mammalia as well as the degradation of xenobiotics in bacteria (Kumar, 2010; Kumar et al., 2012). Sakaki exemplarily describes the lab-scale metabolization of dioxine by rat CYP1A1 along with the possibility to use this pathway in microbiological approaches for the bioremediation of contaminated soil (Sakaki et al., 2013). Generally, the self-sufficient CYP102A1 (also known as P450_{BM3}) is the most investigated P450, exhibiting the highest turnover numbers of all P450s and therefore serving as a model for optimization studies (Gillam, 2008; McIntosh et al., 2014). Other self-sufficient P450s are present in extremophiles in polluted sites, as they show extraordinary stability in harsh conditions (Correddu et al., 2021) and are furthermore described in biotechnological approaches for the chemical and pharmaceutical industry (Ciaramella et al., 2017).

Addressing whole-cell conversions, an alternative to the *E. coli* whole-cell production system was established in recent years in *B. megaterium*. It had already been used in the production of e.g. alpha- and beta-amylase (Hebeda et al., 1988; Vihinen & Mantsiila, 1989). Besides the advantages of a high protein production capacity and its cell size (Vary, 1994), *B. megaterium* contains particular P450s that show catalytic activity towards terpenes, fatty acids, and additional hydrophobic substances. For example, cytochromes of the CYP106-family present in *B. megaterium* show selective hydroxylation activity using steroid hormones (Kiss et al., 2015; Putkaradze et al., 2017; Schmitz et al., 2014) and terpenes like abietic acid and 11-ketoboswellic acid (Bleif et al., 2011, 2012; Janocha et al., 2015). The whole-cell catalysts may take

over a significant role in the industrial production process for steroidal compounds as they offer highly selective modifications, low waste conversions, and environmentally friendly conditions.

1.5 P450 redox partners in general

P450s usually need other proteins as redox partners, which take over the electrons from the NADPH and transfer them to the P450 (Ortiz de Montellano, 2005), as seen in the catalytic cycle in Figure 1: General reaction of cytochrome P450 systems

. These redox partners are diverse and have been classified into ten classes of electron transfer chains (Hannemann et al., 2007), which can occur as individual proteins or various versions of fusion proteins. Out of these ten classes, the classes I and II are the most common ones (see Figure 3). Class I consists of three single proteins: a FAD-containing reductase which is soluble in bacterial systems or associated with the inner mitochondrial membrane in eukaryotes, a soluble ferredoxin of the type [2Fe-2S] as electron shuttle, and a P450, which is soluble for bacterial systems and bound to the inner mitochondrial membrane in eukaryotes. Most bacterial and mitochondrial P450s together with their redox partners belong to the class I. In a first step, the electrons from the redox cofactor are transferred onto the ferredoxin reductase, while $\text{NAD(P)} + \text{H}^+$ is oxidized to NAD(P)^+ . The reductase enables the sequential transfer of the electrons using the ferredoxin to transport the electrons to the P450. Redox systems of class II have two single proteins (see Figure 3C): a P450 and a NADPH-cytochrome-P450 reductase (CPR) containing the prosthetic groups FAD and FMN, where the additional FMN takes over the task to transfer the electron to the P450 instead of the ferredoxin. In case of class II, both proteins are bound to the membrane of the endoplasmatic reticulum (ER).

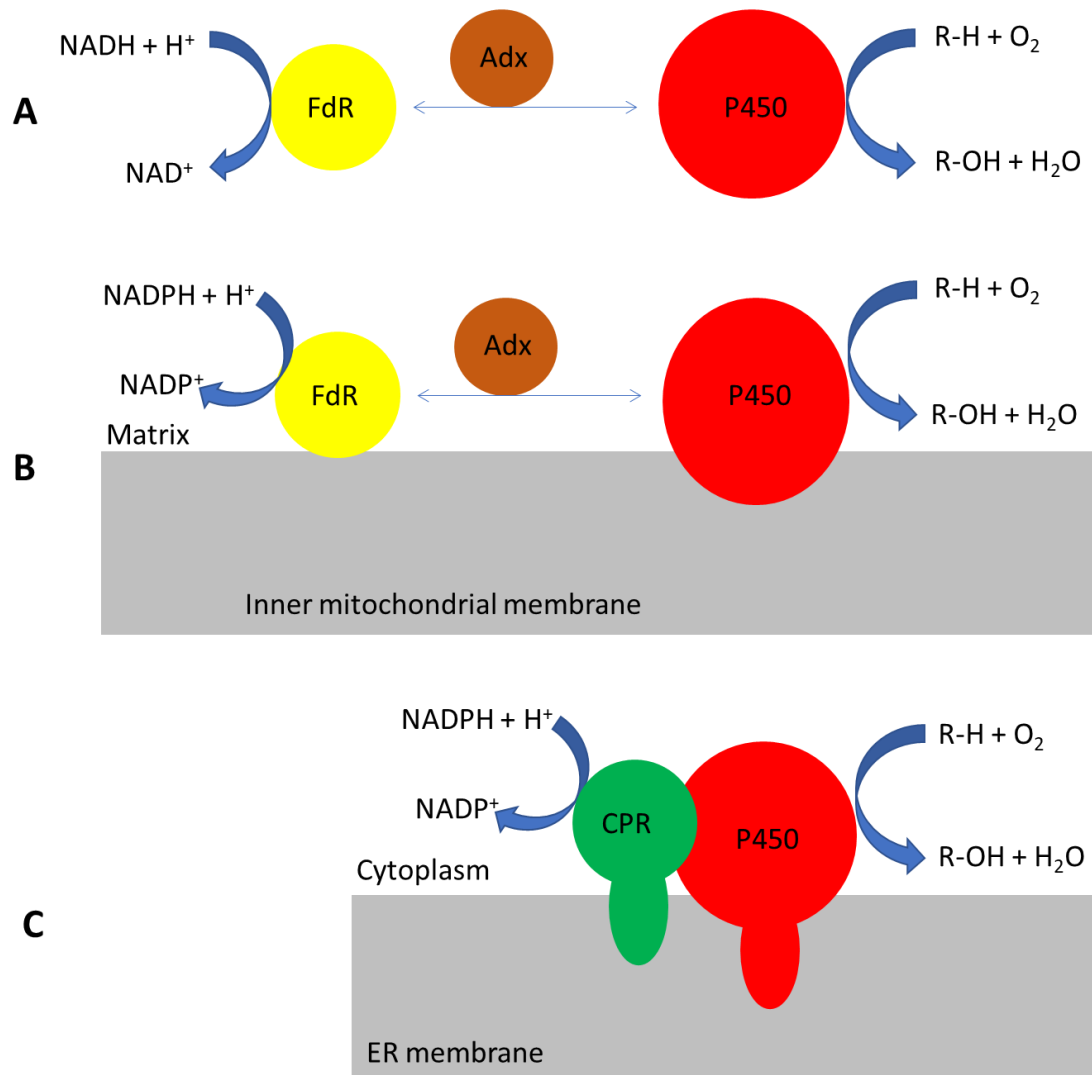


Figure 3: Overview of the two most common classes of redox systems in P450s (modified from (Hannemann et al., 2007)),
A: class I containing three proteins being FAD-containing reductase, ferredoxin as electron shuttle, and the P450 (all soluble in bacterial systems); **B** class I containing three proteins being FAD-containing reductase and the P450 (both bound to mitochondrial membrane in eucaryotes) as well as the soluble ferredoxin as electron shuttle; **C:** class II containing two proteins being the P450 and the CPR (both bound to the ER membrane)

Since the homologous redox partners of bacterial P450s are rarely known or are difficult to express, heterologous redox partners are frequently used. Especially in *in vitro* experiments, the expressibility and, accordingly, the availability are important aspects. Redox proteins with a wide variety of properties, such as different redox potentials, have been discovered and modified over the years. Therefore, excellently matching partners can be found for numerous known bacterial P450s, which enable high electron transfer. Yet moreover, the dependence on redox partners is an important limiting factor for the biocatalytic utilization of cytochrome P450s. Nevertheless, as soon as new P450s are discovered, a basic task is the search for their corresponding redox partners, preferably the natural ones, which can be hard, because the corresponding genes might not be closely localized (Parajuli et al., 2004). With the elucidation

of the crystal structure of redox proteins around the 2000s (Müller et al., 1998; Müller et al., 1999, 2001, 2011; Pikuleva et al., 2000; Ziegler et al., 1999; Ziegler & Schulz, 2000), the binding of the P450 and redox partners was also closely examined to analyze their influence on the electron transfer. Redox processes can be divided into different steps, such as the binding of the proteins, the protein rearrangements, and the transfer of the electrons. The protein surfaces are a critical point for the binding of the proteins. The modification of areas in the redox system, which hinder the complex binding to the P450, could lead to easier modifications of the product pattern than the modification of the P450 itself, as this influences binding and, therefore, the electron transfer velocity, too.

1.6 CYP106A2 from *Bacillus megaterium* (*B. megaterium*)

CYP106A2 is a cytochrome P450 monooxygenase from *B. megaterium* ATCC 13368. It is soluble, 47 kD in size, and consists of 410 amino acids. The native substrate of CYP106A2 is still unknown as is its biological function. As CYP106A2 selectively hydroxylates 3-oxo- Δ^4 steroids in the 15 β position, it is also named 15 β -hydroxylase (Schmitz et al., 2018). McAleer discovered this activity in a strain of *B. megaterium* (McAleer et al., 1958), but only in 1975 Berg was able to prove that the reaction is cytochrome P450-dependent (Berg et al., 1975). Subsequently, Berg resolved the hydroxylase system into the three proteins, megaredoxin reductase, megaredoxin and P-450meg (Figure 4), the latter one being his description of the P450 now known as CYP106A2. Berg also showed the conversion of substrates like 4-androstene-3,17-dione, testosterone, progesterone, and several additional ones (Berg et al., 1976).

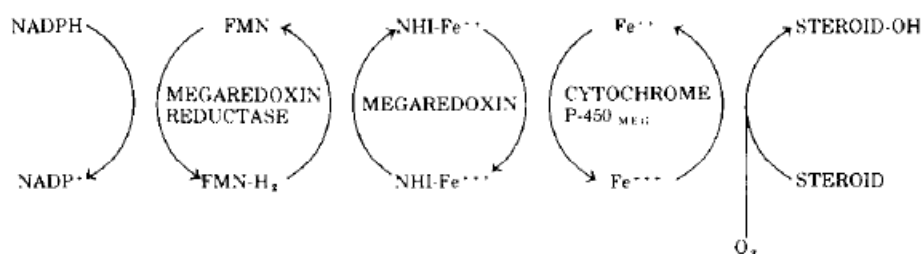


Figure 4: Electron transport in the steroid hydroxylase system of *B. megaterium* (Berg et al., 1976))

After the purification of P-450meg, it was proven that the megaredoxin and megaredoxin reductase can be replaced by adrenodoxin (Adx) and adrenodoxin reductase (AdR) without significant impairment of the steroid hydroxylation (Berg & Ingelman-Sundberg, 1979). These two original redox partners of P-450meg have been characterized (Berg, 1982), but have not

been cloned yet. As the mammalian Adx and AdR are able to provide the necessary electrons to CYP106A2, it was well studied afterwards by Rauschenbach, who cloned and expressed the P450 (Rauschenbach et al., 1993). They presented as well the fact that redox systems of *Bacillus subtilis* (*B. subtilis*) can transfer electrons towards CYP106A2, too. Generally, it was observed that CYP106A2 can utilize several redox partners to reconstitute the redox systems: Besides the already mentioned Adx and AdR, putidaredoxin and putidaredoxin-reductase work as well (Berg et al., 1976; Rauschenbach et al., 1993; Simgen et al., 2000). The bovine systems of Adx4-108 and AdR are best characterized, with Adx4-108 having a lower redox potential than wild-type Adx (Uhlmann et al., 1997) and therefore resulting in better reduction of CYP106A2 (Hannemann et al., 2006).

The substrates CYP106A2 is able to metabolize are numerous and include di- and triterpenes like abietic acid (Janocha et al., 2016), and 11-keto- β -boswellic acid (Bleif et al., 2012), as well as steroids, like deoxycorticosterone and progesterone (Schmitz et al., 2018), which have been extensively investigated in our laboratory. Especially the conversion of the model substrate progesterone was studied in more detail. Various mutations to CYP106A2 have been constructed via saturation mutagenesis and rational site-directed mutagenesis of active site residues to tune the product pattern of the hydroxylation of progesterone towards higher yields of the side products 6 β -, 9 α -, and 11 α -hydroxyprogesterone (Nguyen et al., 2012; Nikolaus et al., 2017). These side products of the progesterone conversion from the wild-type can be employed as pharmaceuticals, especially in anti-androgenic and blood-pressure regulation drugs, or as useful intermediates to produce active drug compounds (Kang et al., 2004; Nguyen et al., 2012). In addition, di- and polyhydroxylated products of progesterone were also described and they lower the overall yield of the conversion (Lisurek et al., 2004; Virus et al., 2006). The rational design of the mutants of CYP106A2 was based first on the homology model developed by Lisurek (Lisurek et al., 2008; Virus et al., 2006) as no crystal structure was available. In 2016, Janocha was ultimately able to solve the crystal structure of CYP106A2 with a resolution of 1.8 and 2.4 Å in the substrate-free and abietic acid bound enzyme, respectively (Janocha et al., 2016). These structural data were used as additional basis for subsequent investigations in our lab (Nikolaus et al., 2017; Putkaradze et al., 2017; Putkaradze et al., 2017; Sagadin et al., 2018).

In-depth research was conducted to address the output of CYP106A2 in whole-cell systems: expression on the outer membrane of *E. coli* cells by Autodisplay (Schumacher et al., 2012), screening of a natural product library of 502 compounds (Schmitz et al., 2012), use of different

bacterial expression strains (Bleif et al., 2011; Schmitz et al., 2014), or upscaling of conversion volume via the use of a bioreactor (Kiss et al., 2015). Moreover, the use of different redox partner systems *in vitro* has been investigated (Goñi et al., 2009; Sagadin et al., 2018).

1.7 Ferredoxins, especially adrenodoxin

Ferredoxins are small electron carrier proteins with at least one iron-sulfur cluster. The number and type of the iron-sulfur clusters leads to different classes, wherein e.g. the [2Fe-2S] class is divided into three subfamilies: plant-type, thioredoxin-like, and vertebrate-type. The latter one includes Adx and Pdx (bacterial putidaredoxin from *Pseudomonas putida* (*P. putida*)), the best studied so far, both being involved in the electron transfer chain of P450s (Ewen et al., 2011), as well as the Etp1^{fd} (electron transfer protein 1 ferredoxin domain) from *Schizosaccharomyces pombe* (*S. pombe*), which has been discovered in our lab as well (Bureik et al., 2002; Schiffler et al., 2004). Ferredoxins of the [2Fe-2S]-type display a conserved hydrophobic core domain with an iron-sulfur cluster and a highly flexible interaction domain for proper specific binding to the partners (reductase and P450) (Holden et al., 1994). They can accept and donate electrons because of their low redox potential which underlines their electron transfer function in all trees of life at very different reactions (Meyer, 2000, 2008). Truncation of ferredoxins are a possibility to adapt a heterologous electron transfer protein to their new "host" as some truncations allow a closer "hugging" of two involved proteins and, therefore, a better swap of electrons (Müller et al., 1998), as well as induce a stronger binding of the cofactor than wild-type.

Adx is localized in the mitochondria of adrenal glands to transfer electrons to the mitochondrial P450s of the steroid metabolism (Bernhardt, 2006; Ewen et al., 2011). It contains 128 amino acids and is of low molecular weight (14.4 kDa). The truncated form Adx4-108 was the first one to be crystallized (A. Müller et al., 1998), showing a (α + β) protein of five β strands and five helices, forming a core domain (aa 5-55 + aa 91-108) and a large hairpin (aa 56-90), the latter serving as interaction domain towards AdR and P450. The crystallization of the full-length (wild-type) Adx was accomplished in 2000 (Pikuleva et al., 2000). Especially the highly conserved proline at position 108 is essential for the correct protein folding and incorporation of the iron-sulfur cluster, with a deletion resulting in a non-active enzyme *in vivo* (Grinberg et al., 2000; Grinberg & Bernhardt, 1998; Uhlmann et al., 1994). Also, further amino acid mutations (e.g. on positions Arg14, Ile58, His56, Tyr82, Thr54, Thr49) show the importance of

hydrogen bonds and salt bridges for the stability of the protein (Burova et al., 1996; Grinberg et al., 2000; Hannemann et al., 2001; Pikuleva et al., 2000; Uhlmann & Bernhardt, 1995). The contact sites to the AdR and P450 are residues exposed on the flexible interaction domain surface, namely Asp76 and Asp79 as well as Asp72 and Glu73, respectively (Coghlan & Vickery, 1992; Vickery, 1997). This kind of clustered occurrence of contact amino acids supports the electron shuttle function of Adx, as it would not be possible for both, AdR and P450, to bind to the same small surface at the same time.

As human P450s displayed activity in *S. pombe* without Adx being co-expressed, the existence of an electron transfer protein from *S. pombe* was discovered (Bureik et al., 2002). The N-terminus showed a high homology to Adx (Bureik et al., 2002), whereas the C-terminus resembled the COX15 protein from *S. cerevisiae* (Glerum et al., 1997). If overexpressed in *S. pombe*, a higher conversion of DOC and RSS was observed than without the overexpression, a fact that was supported by the heterologous expression of the N-terminal protein part in *E. coli* and subsequent use *in vitro* in a conversion assay with AdR and bovine CYP11B1 as well as bacterial CYP106A2 (Bureik et al., 2002). The N-terminal protein part was named electron transfer protein 1 ferredoxin domain (Etp1^{fd}) and was further characterized by Schiffler (Schiffler et al., 2004). Etp1^{fd} exhibits a UV/Vis spectrum similar to that of Adx, whereas the redox potential was determined to be -353 mV instead of -273 mV for Adx. Nevertheless, Etp1^{fd} can replace Adx as electron carrier towards several CYPs (CYP11B1, CYP11A1, CYP106A2, CYP105A1) as well as cytochrome c (Kleser et al., 2012; Schiffler et al., 2004). The three-dimensional crystal structure of Etp1^{fd} has been determined, showing a sequence similarity of 39 % towards Adx, but also revealing a melting temperature more than 20 °C higher than Adx (Müller et al., 2011).

To complete this section about electron transfer proteins, one has to mention flavodoxins as well. They can also take over the function of ferredoxins and contain the non-covalently bound FMN. Flavodoxins transfer the electrons to the corresponding P450 in the organism itself as well as in *in vivo* and *in vitro* conversion (Jenkins & Waterman, 1994; Kimmich et al., 2007).

1.8 Reductases, especially adrenodoxin reductase

AdR is a NADPH-dependent enzyme with the prosthetic group FAD, discovered in 1966 (Nakamura et al., 1966; Omura et al., 1966). The crystal structure was solved in 1999 (Ziegler et al., 1999). Associated at the inner mitochondrial membrane (Hanukoglu et al., 1990; Ishimura

& Fujita, 1997), AdR serves as electron donor to Adx and completes the electron transfer chain between NADPH-AdR-Adx-P450 (Grinberg et al., 2000). AdR converts the two-electron-package from NADPH to two single electrons, which are sequentially transferred via Adx to the P450.

Bovine AdR comprises 460 aa and has a molecular weight of 50.3 kDa, divided into the FAD-containing domain and the NADPH-binding domain, mediating between NADPH and the Adx. The remarkable asymmetric charge distribution on the surface of the interaction area of AdR most likely controls the approach and binding of Adx and, therefore, also the electron transfer (Ziegler et al., 1999).

The putative analogous ferredoxin reductase Arh1 of *S. pombe* is able to replace the AdR used in former conversion assays with Etp1^{fd} and can act as an autologous electron donor to Etp1^{fd} (Bureik et al., 2002; Ewen et al., 2008). Its existence was postulated by Bureik based on an open reading frame in the *S. pombe* genome with high homology to the AdR (Bureik et al., 2002). Subsequently, the gene was cloned, expressed, and purified by Ewen (Ewen et al., 2008). The weak FAD-binding of the wild-type Arh1 was modified by site-directed mutagenesis (arh1_A18G) resulting in a more stable protein which shows similar spectroscopic features to AdR and a molecular weight of 51.4 kDa (Ewen et al., 2008).

1.9 Aim of the Work

The aim of the work was to develop strategies for a “universal” electron transfer module to support P450s, which could facilitate their industrial and biotechnological use.

This Thesis focused on optimizing the redox partners of CYP106A2 to enhance product formation. Several combinations of redox partners were evaluated to identify and optimize the best fit for maximizing main product yields. Bioinformatical approaches, such as molecular protein-protein docking, were employed to explain the underlying causes for these small but distinctive structural changes. Furthermore, these methods guided the modification of the involved ferredoxin to make it an even better fit regarding the conversion of progesterone.

Another goal was to design functional fusion proteins using different types of linkers. These constructs aimed to stabilize the system, facilitate purification processes, and the establish of a “cage” concept. The latter was carried out by integrating the NADPH-regenerating enzyme alcohol dehydrogenase (LB-ADH) from *Lactobacillus brevis* directly into the redox modules,

Introduction

so that cofactors are continuously regenerated in close proximity to the enzyme, thereby reducing the need for expensive external additions.

The final goal was to co-immobilize the different redox partners, and if possible, also the fusion proteins on modified mesoporous silica (MCF) to enable efficient, regio- and stereoselective hydroxylation.

2 Scientific articles

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

2.1 Sagadin et al. (2018)

Binding modes of CYP106A2 redox partners determine differences in progesterone hydroxylation product pattern

Tanja Sagadin, Jan L. Riehm, Mohammed Milhim, Michael C. Hutter, Rita Bernhardt

Communications Biology, (2018) 1:99, DOI: 10.1038/s42003-018-0104-9



ARTICLE

DOI: 10.1038/s42003-018-0104-9

OPEN

Binding modes of CYP106A2 redox partners determine differences in progesterone hydroxylation product patterns

Tanja Sagadin¹, Jan L. Riehm², Mohammed Milhim¹, Michael C. Hutter² & Rita Bernhardt¹

Natural redox partners of bacterial cytochrome P450s (P450s) are mostly unknown. Therefore, substrate conversions are performed with heterologous redox partners; in the case of CYP106A2 from *Bacillus megaterium* ATCC 13368, bovine adrenodoxin (Adx) and adrenodoxin reductase (AdR). Our aim was to optimize the redox system for CYP106A2 for improved product formation by testing 11 different combinations of redox partners. We found that electron transfer protein 1(516–618) showed the highest yield of the main product, 15 β -hydroxyprogesterone, and, furthermore, produced a reduced amount of unwanted polyhydroxylated side products. Molecular protein–protein docking indicated that this is caused by subtle structural changes leading to alternative binding modes of both redox enzymes. Stopped-flow measurements analyzing the CYP106A2 reduction and showing substantial differences in the apparent rate constants supported this conclusion. The study provides for the first time to our knowledge rational explanations for differences in product patterns of a cytochrome P450 caused by difference in the binding mode of the redox partners.

¹Institute of Biochemistry, Campus B2.2, 66123 Saarbrücken, Germany. ²Center for Bioinformatics, Campus E2. 1, 66123 Saarbrücken, Germany. Correspondence and requests for materials should be addressed to R.B. (email: ritabern@mx.uni-saarland.de)

CYP106A2 is a bacterial cytochrome P450 (P450) from *Bacillus megaterium* ATCC 13368 that is able to use a wide range of biotechnologically important substances as substrates, for example, steroids and terpenes^{1–10}. Like almost all P450s, it requires electron transfer proteins to transmit electrons from NAD(P)H to the substrate¹¹. A redox system as part of the 15 β -hydroxylase system from *B. megaterium*, consisting of a NADPH-dependent flavoprotein and an iron-sulfur protein (designated as megaredoxin reductase and megaredoxin, respectively), has been purified and shown to provide the CYP106A2 with electrons, but has never been cloned^{2,12}. It was later reported that CYP106A2 can also accept electrons from the adrenal redox system, comprising adrenodoxin (Adx) and adrenodoxin reductase (AdR)^{13,14}. More research finally led to the use of the truncated version of Adx, Adx(4–108)^{15,16}. Since genome mining of the *B. megaterium* strain DSM319, which is closely related to ATCC 13368, revealed a novel P450, CYP106A1 (showing 63% sequence identity towards CYP106A2), several ferredoxins as well as one flavodoxin, which support the activity of CYP106A1, it was possible to present the first cloned natural redox partners of P450s from *B. megaterium*¹⁷. Nevertheless, the widely used system to work with CYP106A2 in vitro as well as in vivo is the heterologous electron transfer system consisting of the soluble bovine Adx and the membrane-associated bovine AdR^{5–7,13–15,18}. Adx and AdR are localized in adrenal mitochondria and realize the electron transfer to the mitochondrial cytochromes P450 CYP11A1, CYP11B1, and CYP11B2¹⁹. Both proteins are more or less easy to express and to purify and are able to efficiently provide electrons to CYP106A2 to convert all known substrates.

Optimization of the interaction between the redox partners and CYP106A2 could be a major advantage to conduct faster substrate conversions and to obtain higher product yields. Thus, sets of Adx mutants have been previously constructed to modify the interaction parameters with their natural redox partners from adrenal mitochondria, CYP11A1, CYP11B1, and CYP11B2. Truncation of the C-terminus by the removal of 20 amino acids, resulting in the mutant Adx(4–108), was able to accelerate the first step of the electron transfer to CYP11B1 up to 4.5-fold. Also a decreased K_m value and increased V_{max} value was displayed when converting 11-deoxycorticosterone by CYP11B1²⁰. A further truncation by one amino acid to Adx(4–107), however, led to a poor expression of the protein²⁰, indicating that the highly conserved proline at position 108 is essential for correct protein folding and incorporation of the [2Fe-2S] cluster, a fact that was verified by the elucidation of the crystal structure of Adx(4–108)²¹. The exchange of the serine at position 112 by tryptophan (S112W) and the cleavage of the 16 remaining C-terminal amino acids lowered the redox potential by 64 mV (–334 mV) compared to the wild type (–270 mV). Furthermore, Adx (S112W) showed increased k_{cat} values and lowered K_m values for the conversion of cholesterol to pregnenolone by CYP11A1²².

However, besides the replacement of the electron transfer protein Adx by mutants of Adx, the exchange with redox systems of other than mammalian origin may provide opportunities to modify the interactions between CYP106A2 and the redox partners. Therefore, we studied the influence of different redox systems on the progesterone conversion catalyzed by CYP106A2. Eleven different combinations of redox partners were tested for conversion and product pattern and included (besides Adx and AdR) the ferredoxins Adx(4–108), electron transfer protein 1 (Etp1)(516–618) (*Schizosaccharomyces pombe*), Fdx2 (*B. megaterium*), the flavodoxins FldA (*Escherichia coli*), and YkuN (*Bacillus subtilis*) as well as the reductases AdR homolog 1 (Arh1) (*S. pombe*), BmCPR (*B. megaterium*), and Fpr (*Escherichia coli*). These redox partners were chosen as they are well studied and characterized in detail. They serve

as electron donors in vitro as well as in vivo to a range of bacterial and mammalian P450s, such as CYP106A2²³, CYP106A1²⁴, CYP105A1²⁵, CYP109B1^{26,27}, CYP267B1²⁸, CYP264A1²⁹, CYP11A1³⁰, and CYP21A2^{24,31}. The model substrate progesterone is a well-studied substrate of CYP106A2. Its main product is 15 β -hydroxyprogesterone (15 β -OH-P). Other monohydroxylated products are 6 β -hydroxyprogesterone, 9 α -hydroxyprogesterone, and 11 α -hydroxyprogesterone, which, however, appear in low quantities^{15,32}. In addition to the monohydroxylated progesterone products, polyhydroxylated progesterone products are also occurring, which lower the overall amount of monohydroxylated products.

We were able to demonstrate the strong influence of redox partner choice not only on the rate of substrate conversion but also on the product pattern of the multi-product conversion of progesterone by CYP106A2. The most promising ferredoxin to achieve a high yield of the main product 15 β -OH-P, while reducing the amount of unwanted polyhydroxylated progesterone products, proved to be Etp1(516–618) from *S. pombe*. Using molecular docking and stopped-flow measurements, we were able to present a rational explanation for this phenomenon.

Results

Conversion of 30 min with 11 redox partner combinations. To test the ability of the various redox chains for their efficiency to transfer electrons from NAD(P)H to CYP106A2, in vitro experiments using various heterologous redox systems were conducted. The typically used redox system for CYP106A2 is the truncated form of bovine Adx, Adx(4–108), and the corresponding reductase, AdR, which was therefore used as the “parent” system. We investigated different naturally occurring redox protein systems as well as combinations between them. The redox system of *S. pombe*, consisting of the ferredoxin Etp1 and ferredoxin reductase Arh1, is completely soluble and easily expressible^{33,34}. With the bacterial CYP105A1 from *Streptomyces griseolus*, the reduction efficiency was shown to be 100% for the combination of Etp1/Arh1, compared to only ~25% when using Adx(4–108)/AdR²⁵. Other possibilities for reconstitution are the full-length bovine Adx, which works better with CYP11A1³⁵ than the truncated form (Adx(4–108)) that is mostly used for CYP106A2, as well as redox partners from different *Bacillus* strains, such as the ferredoxin isolated from *B. megaterium* DSM319, Fdx2^[117], the typical short-chain flavodoxin YkuN from *B. subtilis*³⁶, the *B. megaterium* P450 reductase BmCPR²⁴, or redox partners from *E. coli*, for example, the flavodoxin FldA and NADPH-dependent flavodoxin/ferredoxin reductase Fpr^{26,27,29} (Fig. 1). These redox partners were selected as they are well studied and characterized before, and used for very different P450 species of bacterial and mammalian origin that are known to accept these redox proteins as electron donors in vitro as well as in vivo. The driving force for the ability to transfer electrons could be the redox potential (e.g., Etp1(516–618) displays a redox potential of -381 ± 2 mV³⁷ compared with -344 mV³⁸ for Adx(4–108)) as well as the differences in the binding mode of the redox partners to CYP106A2. Here, we focused on the interaction between both proteins and analyzed the product yield as well as the product pattern.

First tests were conducted with a fixed ratio of P450:ferredoxin: reductase of 1:20:2 (exceptions for P450:FldA:Fpr = 1:50:50 and P450:YkuN:Fpr = 1:10:10) for 30 min to ensure the compatibility of redox proteins with CYP106A2 (Fig. 2).

Calculating the 15-hydroxylase activities of CYP106A2, it is obvious that the redox partner sets achieve different activities (Table 1). For example, for the use of Etp1(516–618), the activities are doubled or nearly tripled compared to those of Adx

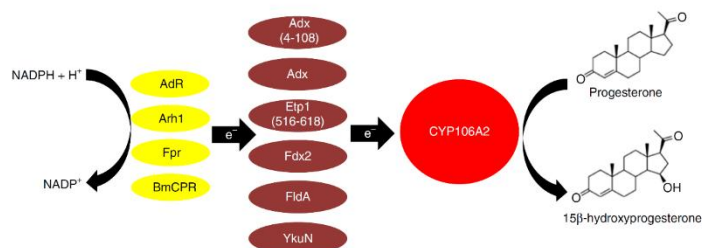


Fig. 1 Schematic drawing of the investigated redox partner proteins. Redox equivalents are transferred from NADPH via a reductase (AdR, Arh1, Fpr, BmCPR) and a ferredoxin (Adx, Adx(4–108), Etp1(516–618), Fdx2), or flavodoxin (FldA, YkuN)) to the progesterone converting enzyme, CYP106A2

(4–108). Most interestingly, the product patterns of the progesterone conversion differ clearly between the tested combinations of redox partners (Fig. 3). Some combinations have similar overall conversion rates but produce more than 60% polyhydroxylated progesterones (e.g., Adx(4–108)/Arh1), whereas others yield more than 70% 15 β -OH-P (Etp1(516–618)/AdR). Also, redox partner combinations which have slower conversion rates are distinguishable (Adx/AdR). Using the above-mentioned conditions (ratio of enzymes, temperature, time, in vitro components), it is clearly visible that the ferredoxin Adx(4–108) (combined with AdR) produces a substantial amount of polyhydroxylated products, whereas the wild-type Adx, as well as the FldA, show a lower overall conversion of progesterone (Fig. 2). Etp1(516–618) demonstrates a very high conversion (similar to Adx(4–108)/AdR) but a low amount of polyhydroxylated progesterone products. The combination of Adx(4–108) and BmCPR displays a high conversion with also low amounts of polyhydroxylated products.

All combinations (except FldA/Fpr) tested did not remarkably change the amount of the other monohydroxylated progesterone products (6 β -hydroxyprogesterone, 9 α -hydroxyprogesterone, and 11 α -hydroxyprogesterone), which always was between 3 and 5% under the conditions being tested. Switching the established bovine reductase, AdR, to the yeast Arh1 resulted in only little changes: 27% of the total product consists of the main product, 15 β -OH-P, when using AdR as reductase, and 31% when using Arh1. Also, a difference in polyhydroxylated progesterone was not notable, which is 65 and 64% when using AdR and Arh1, respectively. When replacing the ferredoxin from Adx(4–108) to Etp1(516–618), the change in the product distribution is remarkable. The combination of Etp1(516–618) and AdR resulted in 78% main product and only 18% polyhydroxylated progesterone, which is a 2.6-fold increase in the main product when compared to Adx(4–108)/AdR. Also the source of reductase seems to affect the product pattern. The use of Arh1 resulted in 66% main product and 26% poly-OH-progesterone compared to 78 and 18% when AdR was used (Fig. 2). Applying the full-length Adx(1–128) completely changes the product formation: there are still 41 and 36% of the substrate left (in combination with AdR and Arh1, respectively), but there are also 53 and 58% of 15 β -OH-P formed. The amount of polyhydroxylated products is negligible. The use of Fdx2 from *B. megaterium* resulted in 66% main product and 29% of unconverted substrate. No polyhydroxylated products were formed. In contrast, the redox partners originating from *E. coli*, FldA and Fpr, were not able to provide electrons to CYP106A2 when a ratio of P450:ferredoxin:reductase of 1:20:2 was applied, which resulted in no product formation. Therefore, the ratio was increased to 1:50:50, which led to a conversion of 27% (all main product). Exchanging the FldA with the YkuN from *B. subtilis* (at a ratio of 1:10:10), a production of

24% poly-OH-progesterone and 63% main product was observed. Surprisingly, the use of the very recently cloned and characterized *B. megaterium* reductase BmCPR²⁴ resulted in 80 and 72% 15 β -OH-P, when combined with Adx(4–108) and Etp1(516–618), and only 10 and 4% poly-OH-P formation, respectively. These data demonstrate that the application of different redox systems can alter not only the conversion rate of the substrate but also the product distribution.

Time-course measurements for four redox partner combinations. To gain deeper insight into the time dependence of the progesterone conversion and product distribution by CYP106A2, the combinations of the ferredoxins Adx(4–108) and Etp1(516–618) with AdR and Arh1 were investigated in more detail. When using Adx(4–108)/AdR (Fig. 4), the steep increase of 15 β -OH-P formation up to 67% (5 min) is followed by a decrease to 34% after 60 min. At the same time, the amount of polyhydroxylated progesterone is slowly increasing from 7% after 5 min to 59% after 60 min. This suggests that the main product is partially further hydroxylated to result in polyhydroxylated end products. Within 10 min, 93% of progesterone has been converted.

After changing AdR for Arh1, the time dependence of progesterone conversion was mostly identical. The conversion of progesterone is almost complete after 5 min (1% left), whereas the rate of 15 β -OH-P (76%) decreases rapidly after 5 min while increasing amounts of polyhydroxylated progesterones are formed (up to 66%). Using Etp1(516–618) as a ferredoxin with AdR as corresponding reductase, a steep increase of 15 β -OH-P formation to 75% within the first 5 min is followed by a slow increase up to 81% after 60 min. In this case, a slow rise of poly-OH-progesterone (not reducing the amount of the main product) to 14% after 60 min occurs. Within 10 min of conversion, 95% of the progesterone is converted. Changing the reductase for Arh1, the highest main product formation is observed after 10 min (78%), dropping slightly to 69% after 60 min. This is accompanied by an increase of poly-OH-P from 6% after 10 min to 26% after 60 min. Progesterone conversion is almost complete after 15 min (1% left). In all investigated cases, further monohydroxylated products remain at about 4% over the time-course of 60 min.

Protein-protein docking and mutagenesis analysis. Etp1(516–618) from *S. pombe*, which exhibits 81% sequence identity to the shortened, yet fully functional Adx(4–108), is also highly similar in its three-dimensional structure as the comparison of the high-resolution crystal structures shows (Fig. 5). Particularly in the region around the iron-sulfur (FeS) cluster any substantial deviations of the protein backbone are absent. Our docking

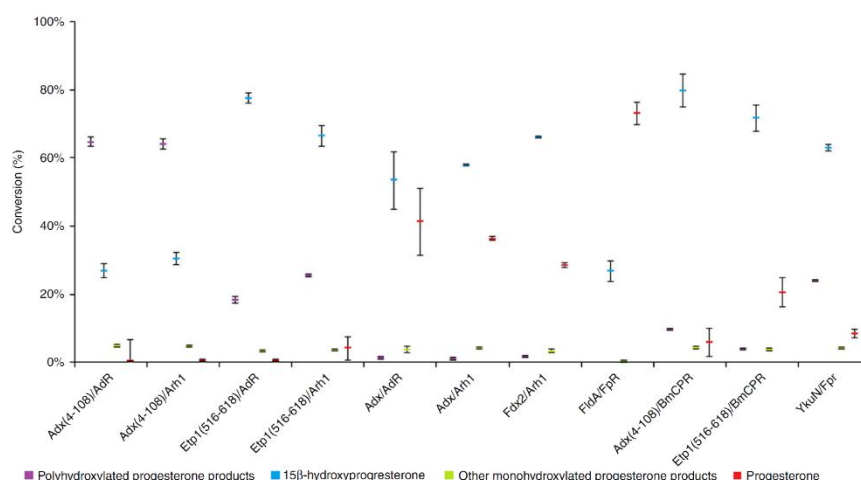


Fig. 2 Comparison of in vitro conversions of progesterone with CYP106A2 and different redox partner combinations. Reactions were run for 30 min while keeping the ratio of P450:ferredoxin:reductase at 1:20:2 (except for P450:FldA/FpR = 1:50:50 and P450:YkuN/FpR = 1:10:10); violet marks indicate polyhydroxylated progesterone products, blue marks indicate the main product 15β-OH-P, green marks indicate other monohydroxylated progesterone products (e.g., 11α-hydroxyprogesterone, 9α-hydroxyprogesterone, and 6β-hydroxyprogesterone) (Fig. 3), and orange marks indicate the remaining substrate progesterone. The relative particular product level was calculated by using the relative peak area of the specific product compared to the total peak areas of reactant and products

studies, however, showed that subtle structural differences in the Adx(4–108) prevent it from adopting the same binding orientation to the CYP106A2 as it was found for Etp1 (Fig. 6). It turned out that the residues around positions 14 and 41 of Adx would sterically clash with the cytochrome. Furthermore, Tyr82 cannot form hydrogen bonds to the cytochrome, whereas phenylalanine in the corresponding position of Etp1 forms hydrophobic contacts, whereby penetrating deeply through the cytochrome's surface. There, residues Phe107, Pro359, and Leu356 encase the side chain of Phe82 (Fig. 7). As a consequence, the distance between the heme-iron of the cytochrome and the FeS cluster is substantially shorter in the complex with Etp1 (19 Å) than in that with Adx(4–108) (24 Å).

To confirm that F82 of Etp1(516–618) plays an important role in the interaction and binding between Etp1(516–618) and the CYP106A2, we conducted site-directed mutagenesis studies after calculating the effect of possible mutations. The mutant F82R of Etp1(516–618) was expected to have severe effects on this interaction and was, therefore, constructed, expressed, and purified. Using this mutant, a 47% loss in the overall product formation was observed compared with the wild-type Etp1(516–618). This clearly demonstrates that the position 82 is important for the interaction of Etp1(516–618) with CYP106A2 and that mutations at this site change the binding between both proteins resulting in changed turnover numbers.

The experimentally observed hydroxylation pattern of progesterone, in particular the formation of polyhydroxylated products, requires the reorientation of monohydroxylated progesterone. In order to dissect this process of reorientation from that of the electron transfer by the ferredoxins, we performed molecular dynamics simulation of 15β-OH-P in CYP106A2. The corresponding results show that reorientation of the monohydroxylated product occurs within <100 ns. These molecular movements lead to conformations in which hydroxylation of other positions (11α-hydroxyprogesterone, 9α-hydroxyprogesterone, and 6β-hydroxyprogesterone) are possible, assuming that a distance of

Table 1 Calculated 15β-hydroxylase activities of CYP106A2 and progesterone

Redox proteins	15β-Hydroxylase activity of CYP106A2
Adx(4-108)/AdR	3.59
Adx(4-108)/Arh1	4.07
Etp1(516-618)/AdR	10.34
Etp1(516-618)/Arh1	8.86
Adx/AdR	7.12
Adx/Arh1	7.72
Fdx2/Arh1	8.84
FldA/FpR	3.57
Adx(4-108)/BmCPR	10.64
Etp1(516-618)/BmCPR	9.56
YkuN/FpR	8.40

The calculated 15β-hydroxylase activities of CYP106A2 and progesterone are shown when CYP106A2 was paired with variable redox partners. Conversion time was 30 min. Activities are given in [nmol 15β-OH-P min⁻¹ nmol CYP106A2⁻¹]

<5 Å between the respective carbon atom and the iron of the heme is sufficiently close for reaction.

Stopped-flow measurements. To validate experimentally the influence of the mode of ferredoxin binding regarding the electron transfer velocity, stopped-flow measurements were performed (Fig. 8). Differences in the reduction traces reflect the different binding behaviors that were indicated by the conversion analysis and molecular docking calculations. The resulting apparent rate constants (k_{app}) were calculated and are presented in Table 2. Applying the redox system of Etp1(516–618)/Arh2, the apparent rate constant is 0.343 s⁻¹, whereas the combination of Adx(4–108) and AdR leads to a lower k_{app} of 0.292 s⁻¹. This is in full agreement with the calculated shorter distance of the active

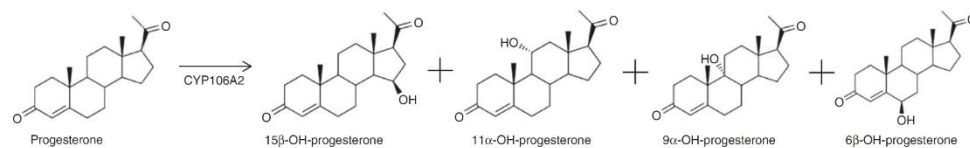


Fig. 3 Conversion of progesterone by CYP106A2 to the monohydroxylated products, 15 β -hydroxyprogesterone, 11 α -hydroxyprogesterone, 9 α -hydroxyprogesterone, and 6 β -hydroxyprogesterone

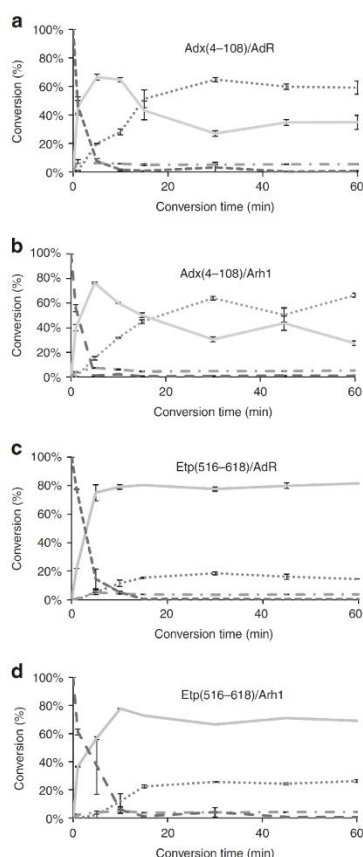


Fig. 4 Time-dependent in vitro conversion of progesterone by CYP106A2 and different redox partner combinations. **a** Adx(4-108)/AdR, **b** Adx(4-108)/Arh1, **c** Etp1(516-618)/AdR, or **d** Etp1(516-618)/Arh1. Conversions were stopped after 1, 5, 10, 15, 30, 45, or 60 min; dotted lines indicate polyhydroxylated progesterone products, gray lines indicate the main product 15 β -OH-P, dashed with single dot lines indicate other monohydroxylated progesterone products, and dashed lines indicate the remaining substrate progesterone

site of the ferredoxin Etp1(516-618) and CYP106A2 compared with Adx(4-108), as well as the faster overall conversion of progesterone when using Etp1(516-618)/Arh1.

Discussion

As every P450 is in need of electrons, the choice of the corresponding redox partners is crucial for the proper function of P450s²⁵. A universal redox system, being able to reconstitute the activity of a majority of P450s, is the ultimate goal of many investigations³⁹. There is a variety of parameters one should consider, for example, expressability, (thermo-) stability, or electron transfer rate.

In this study, we had a closer look at redox systems from different organisms that have already been used with diverse P450s. After identifying an efficient redox system for a specific P450, further optimizations by methods of protein engineering should be performed and finally tests with other P450 isoforms are designated. As a first step to create a universal redox system for P450s, we tested the applicability of diverse redox partners for the well-studied CYP106A2^{6,15,40-42}. The experiments showed clearly that all used combinations of redox partners were able to provide electrons to CYP106A2, but only the truncated forms of Adx and Etp1, combined with AdR or Arh1, were able to support CYP106A2 in a way that almost all provided progesterone (200 μ M) was converted within 30 min.

The most unexpected result was, however, the observation that the exchange of the redox partners resulted in a change of the product pattern. The hydroxylation of progesterone by CYP106A2 reconstituted with the commonly used redox system Adx(4-108) and AdR produces high amounts of polyhydroxylated progesterone products under the conditions studied. When using this “parent” system of Adx(4-108) and AdR for 30 min of conversion, high amounts of polyhydroxylated progesterones and low ones for 15 β -OH-P are observed, a pattern that is dramatically reversed when using Etp1(516-618) (Fig. 2). Investigating the time dependency of the product formation in detail, it was shown that the conversion of progesterone by the ferredoxins is very efficient and complete within 10 to 30 min. Whereas the amount of 15 β -OH-P is rising in every combination within 5 to 10 min, it is afterwards dropping substantially when using Adx(4-108). This harsh decrease is accompanied by a steep increase of poly-OH-P. In contrast, when using Etp1(516-618), the ratio of 15 β -OH-P is either decreasing only slightly after 10 min or remains constant.

Based on these results, it becomes clear that the different redox partner combinations tested here can be used for diverse purposes, depending on the overall product yield and pattern one is aiming for. For approaches going for fast substrate conversions, truncated versions of ferredoxins process most of the substrate within 10 to 15 min. Adx(4-108) would be preferable to those aiming for many polyhydroxylated progesterone products which are of high interest as they represent a high diversity and, therefore, different possibilities for further alteration of the steroid molecule. Since many attempts are focused on high yields of certain monohydroxylated products, application of truncated Etp1(516-618) is useful to obtain high absolute and relative yields of the main product 15 β -OH-P within only 5 min. Systems using

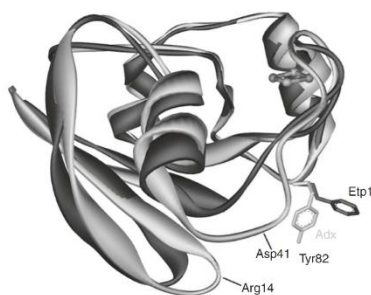


Fig. 5 Superimposition of Adx(4–108) to Etp1(516–618). Superimposition of Adx(4–108) to the obtained docking conformation of Etp1(516–618) (dark gray), which has its FeS cluster in closest distance to CYP106A2. The loops of Adx (light gray) around residues 14 and 41 would clash with the cytochrome (omitted for clarity)

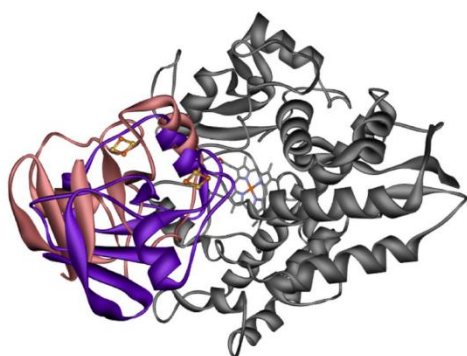


Fig. 6 Overlay of the docking conformations of ferredoxins and CYP. Overlay of the energetically most favorable docking conformations of Adx(4–108) and of Etp1(516–618) to CYP106A2. Adx (coral) is not able to bind in the same orientation as Etp1(516–618) (violet) due to subtle but distinct structural differences

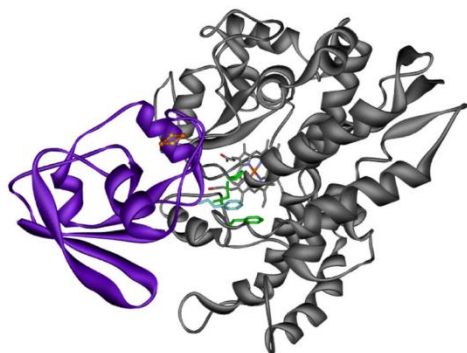


Fig. 7 Docking conformation of Etp1(516–618) and CYP106A2. Obtained docking conformation of Etp1(516–618) (violet) in which its FeS cluster is closest to the heme group of CYP106A2 (gray). Phenylalanine (cyan) penetrates into the cytochrome forming hydrophobic contacts with Phe107, Pro359, and Leu356 (green)

Adx(1–128) as redox partner are a further candidate for the production of monohydroxylated progesterones because they produce very low amounts of polyhydroxylated products. However, in this case the activity is rather low.

A comparison of the ferredoxins as the direct electron transfer proteins to CYP106A2 is of special interest, because the crystal structures of the bovine Adx(4–108) and the yeast Etp1(516–618) have been solved^{21,37}. Both proteins show an amino acid identity of 46% and highly conserved regions. Comparing the obtained complexes from protein–protein docking it becomes obvious that Etp1(516–618) binds closer to CYP106A2 than Adx(4–108) due to steric constraints (see Figs. 5–7). This seems to be caused especially by the presence of F82 of Etp1(516–618). To confirm this hypothesis, we analyzed the effects of potential mutants at this site using computer modeling and examined experimentally the mutant F82R, proposed to have a severe effect on the interaction with CYP106A2. Compared to the wild type, the mutant showed a 47% loss in overall product formation, supporting the proposed importance of the position 82 in the interaction process. The distance between the FeS cluster of the ferredoxins (Adx(4–108) and Etp1(516–618)) and the heme-iron atom is longer (24 Å) for the binding of the Adx(4–108) than for Etp1(516–618) (19 Å). Thus, the closer approach of Etp1(516–618) should allow faster electron transfer. In fact, experimental investigation using the stopped-flow technique to study the electron transfer, revealed differences between the yeast and the adrenal redox system: the apparent rate constant (k_{app}) for the Etp1(516–618)/Arh1 combination (yeast) is 0.343 s^{-1} , while Adx(4–108)/AdR (adrenal) reaches only 0.292 s^{-1} . On the other hand, the binding of Adx(4–108) might be stronger than that of Etp1(516–618), for example, due to larger electrostatic contributions, resulting in a slower release of Adx(4–108). This may lead to a longer disposition of the substrate in the active site of the CYP106A2 and, therefore, also more than one single hydroxylation product. This can be caused by the kind of contact between the ferredoxin and CYP106A2, meaning an intense binding and slow transfer of electrons will not allow the then-build product to exit the active site of CYP106A2, whereas a looser binding and fast electron transfer is enabling the monohydroxylated progesterone to leave the binding pocket before further hydroxylation is performed. In the absence of experimental data on the actual life time of these transient complexes between the respective ferredoxins and CYP106A2, corresponding assumptions must, however, be considered with some care. Nevertheless, our molecular dynamic simulations of 15β -OH-progesterone in the binding pocket of CYP106A2 show that reorientation of the monohydroxylated product occurs within 100 ns, leading to conformations in which hydroxylation of other positions (11 α -hydroxyprogesterone, 9 α -hydroxyprogesterone, and 6 β -hydroxyprogesterone) is likely. This time scale is rather fast compared to that of the product release, which was not observed within the simulation time of 200 ns. Thus, the observed product spectra are governed by several factors, which are not independent of each other.

In summary, our results demonstrate that different aspects play a role when a decision has to be undertaken which redox system is the most preferable one for a certain P450. We were able to show that the use of different redox systems manipulates the rate and the yield of conversion of progesterone by CYP106A2. In addition, the product pattern can be altered between high levels of polyhydroxylated or monohydroxylated progesterone products by changing the redox partners. The application of Etp1(516–618) results in a high quantity of the main monohydroxylated product, whereas Adx(4–108) produces many polyhydroxylated progesterone products. Working with the full-length Adx(1–128), high amounts of monohydroxylated progesterone products are obtained with the disadvantage of slow conversion. These

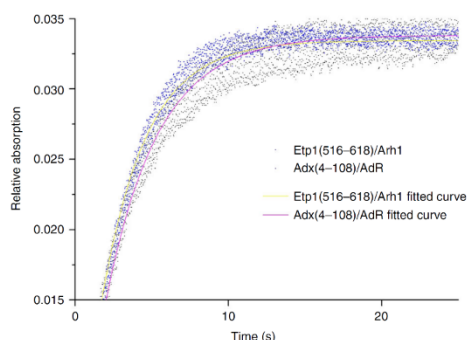


Fig. 8 Averaged curves of stopped-flow measurement. Blue dots represent the Etp1(516-618)/Arh1 redox partner set, and black the Adx(4-108)/AdR set. Yellow and pink lines indicate the fitting, respectively. To analyze the stopped-flow reactions thoroughly, three to four replicates were averaged. The equations used were $f(x) = a \cdot (1 - e^{-b \cdot x})$, b describing the apparent rate constants (k_{app}) for the reduction of CYP106A2 by the two redox partner combinations Adx(4-108)/AdR or Etp1(516-618)/Arh were 0.292 and 0.343 s^{-1} , respectively

Table 2 Apparent rate constants (k_{app}) for the reduction of CYP106A2

Redox combination	k_{app} (s^{-1})
Adx(4-108)/AdR	0.292 $\pm$ 0.001
Etp1(516-618)/Arh	0.343 $\pm$ 0.001

Apparent rate constants (k_{app}) for the reduction of CYP106A2 by the two redox partner combinations Adx(4-108)/AdR or Etp1(516-618)/Arh using 400 μM NADPH, 2 μM ferredoxin reductase (AdR or Arh1), and 20 μM ferredoxin (Adx(4-108) or Etp1(516-618)) in syringe A and 2 μM of CYP106A2 and 200 μM of progesterone in syringe B. Three to four replicates were averaged. The equations used to fit with the data points were: $f(x) = a \cdot (1 - e^{-b \cdot x})$. The given constants correspond to the respective b in the exponent of the equations

experimental data were supported by molecular docking revealing a shorter distance between the FeS cluster of the ferredoxin and the P450 heme for Etp1(516-618) than for Adx(4-108). Backing the molecular docking data, it was shown by stopped-flow measurements that the apparent rate constant (k_{app}) of Etp1(516-618)/Arh1 was higher than that of Adx(4-108)/AdR, therefore resulting in a faster electron transfer between Etp1(516-618) and CYP106A2 than between Adx(4-108) and CYP106A2, explaining the above-mentioned product pattern in conversions with progesterone. Assuming a longer life time of the complex Adx(4-108) with CYP106A2 than Etp1(516-618) with CYP106A2, the monohydroxylated products are able to reorient more often applying Adx(4-108) resulting in more hydroxylations and, therefore, higher amounts of polyhydroxylated products. This knowledge can be employed to modify or tune the amount of the given products. Thus, applying the studied redox systems from bacterial, fungal, and mammalian origin, various effects on the product patterns have been observed and are promising for tuning biotechnological reactions for different purposes.

Methods

Site-directed mutagenesis. The Etp1(516-618) mutant was constructed in the expression vector pTrc99A. The primers (forward primer: GATTTAGCACGTGGTCTCGAGGAACAAGCAG-3 and reverse primer: CTCGAGACACGTGCTCAATCAAGCATGTCTTCTC) used for the site-

directed mutagenesis have been synthesized by Eurofins MWG Operon. The primers were used to construct the mutant Etp1(516-618)/F82R by standard protocols⁴³.

Protein expression in *E. coli* and purification. CYP106A2, Etp1(516-618), and Arh1_A18G were expressed and purified as described elsewhere^{37,15,33,44,45}. Arh1_A18G was used, as it demonstrates a stronger binding of the flavin adenine dinucleotide than the wild-type Arh1³³ (further mentions of Arh1 always refer to the Arh1_A18G mutant). Adx(1-128), Adx(4-108), and AdR were purified as described before^{35,38,46,47}. BmCPR was provided by Mohammed Milhim²⁴. Fdx2 was provided by Elisa Brill¹⁷. FldA, YkuN, and Fpr were provided by Patrick Bakkes and Vlada Urlacher (University of Düsseldorf).

In vitro conversion. A model conversion was performed in potassium phosphate buffer (50 mM, pH 7.4, 0.05% Tween-20) in a total volume of 250 μL with 0.5 μM CYP106A2, 10 μM ferredoxin or flavodoxin, and 1 μM ferredoxin reductase, resulting in a 1:20:2 ratio (P450:Fdx:FdR)⁴⁸. As ferredoxins or flavodoxins, different varieties were employed: bovine Adx, wild-type form (amino acids 1-128, Adx(1-128)) and truncated form (amino acids 4-108, Adx(4-108)), electron transfer protein from *S. pombe* (truncated form, amino acids 516-618, Etp1(516-618)), Fdx2 from *B. megaterium* DSM319¹⁷, YkuN from *B. subtilis*²⁶, and FldA from *E. coli*²⁷. The following enzymes were applied as reductases: bovine AdR, AdR homolog 1 from *S. pombe* (Arh1), BmCPR from *B. megaterium*²⁴ and Fpr from *E. coli*²⁷. 200 μM progesterone was used as substrate and the reaction was started by the addition of 0.1 mM NADPH. A NADPH-regenerating system containing 1 mM $MgCl_2$, 5 mM glucose-6-phosphate, and 4 U mL^{-1} glucose-6-phosphate dehydrogenase was used. The conversion took place at 30 °C and 900 rpm for 1 to 60 min; the reactions were quenched and extracted with 500 μL of ethyl acetate; the organic phases were evaporated to dryness and stored at -20 °C until high-performance liquid chromatography (HPLC) analysis. Three samples were conducted for each tested combination of redox partners.

HPLC analysis. Reversed-phase liquid chromatography was performed using a Jasco system containing an UV-2075Plus Intelligent UV/Vis Detector, an AS-2050Plus Intelligent Sampler, an LG-2080-02 Ternary Gradient Unit, a PU-2080Plus Intelligent HPLC Pump, a DG-2080-53 3-Line Degasser, and an LC-NetII/ADC (Jasco, Gross-Umstadt, Germany). The mobile phase acetonitrile/water 90:10 was degassed prior to use and the flow rate was 1 $mL \cdot min^{-1}$ with the following gradient: a linear gradient for 2 min from 89% solvent A (water containing 10% acetonitrile) to 33% solvent B (100% acetonitrile) holding it for 4 min, a linear gradient for 4 min to 100% solvent B holding it for 2 min, a linear gradient for 0.1 min to 89% solvent A holding it for 3 min. A reversed-phase ac MN Nucleodur C18 (5 μm , 4.0 $\times$ 125 mm^2) column (Macherey-Nagel, Düren, Germany) was adjusted to 40 °C by a column thermostat (W.O. Electronics, Austria). For each measurement, 10 μL of a sample was injected and the absorbance was monitored at 240 nm. The relative product level was evaluated using the relative peak area of the product compared to the combined peak areas of educt and product. The values of the three samples were averaged out (mean average), and standard deviations were calculated.

Computational methods. The homology models of the Adx mutants where constructed applying the corresponding functions of the Swiss-PdbViewer (version 4.01, <http://www.expasy.org/spdbv>) to the crystal structure of bovine Adx (PDB entry code 1AYF, chain B), which comprises residues 5-108²¹. The Adx-like Etp1 was prepared likewise (PDB entry code 2WLB, chain B)³⁷. As receptor, the conformation of CYP106A2 from *B. megaterium* in substrate-bound form was used (PDB entry code 5IKI, chain B). For protein-protein docking the ClusPro2 web service (<https://cluspro.org/login.php>) was used, applying default parameters⁴⁹⁻⁵². Due to the limitations of the underlying method all non protein parts (heme group of the cytochrome as well as the Fe_2S_2 fragment of Adx and of etp1) were removed prior to the docking procedure. For further analysis the complexes obtained according to the „balanced scoring function“ were used. These were inspected manually by superimposing the heme containing structure of CYP106A2 within vmd (version 1.8.6, <http://www.ks.uiuc.edu/Research/vmd/>)⁵³.

Molecular dynamics simulations were carried out on 15 β -OH-progesterone in the binding pocket of CYP106A2 applying GROMACS (version 4.4.5)⁵⁴. The amino acids that were unresolved in the crystal structure (residues 176 to 180) were modeled using the Swiss-PdbViewer. The cytochrome was placed in the center of a cubic box with a minimum distance of 10 Å from the box edge and surrounded by TIP3P waters⁵⁵. Energy minimization was started from the putative conformation of on 15 β -OH-progesterone after hydroxylation. The steepest descent energy minimization was stopped at a gradient norm below 1000 $kJ \cdot mol^{-1} \cdot nm^{-2}$. Equilibration consisted in two different steps: first in a NVT (constant volume, temperature, and number of particles) ensemble for 100 ps maintaining the temperature at 300 K using a modified Berendsen thermostat⁵⁶, and second by stabilizing the system at 1 bar applying a Parinello-Rahman barostat⁵⁷ for 100 ps of NPT (constant pressure, temperature, and number of particles) equilibration. All bonds in both steps were constrained by the LINCS algorithm⁵⁸. Subsequently the production run of 200 ns was performed. Hereby Newton's equations of motion

were integrated by the leapfrog algorithm at time steps of 2 fs. Snapshots of the trajectory were taken at intervals of 1 ps.

Stopped-flow measurements (kinetics by rapid mixing). Stopped-flow measurements were carried out on a SFM 300 stopped-flow spectrophotometer equipped with an FC-15 cuvette and an MPS 60 data-processing unit (Biologic SAS, Claix, France) at 20 °C, as described previously^{22,39}. The system was made anaerobic by repeated flushing with excessively argon-bubbled buffer. The reaction buffer for CYP106A2 consisted of 50 mM HEPES (pH 7.4) and 0.05% Tween-20. Conditions were optimized for the reduction of CYP106A2. All samples were prepared in a glove box in an oxygen-free atmosphere. To follow the whole cascade of reactions within one measurement (i.e., reduction of ferredoxin reductase, ferredoxin, and CYP106A2), syringe A contained 400 µM NADPH, 2 µM ferredoxin reductase (AdR or Arh1), and 20 µM ferredoxin (Adx(4–108) or Etp1(516–618)) and syringe B contained 2 µM CYP106A2 and 200 µM progesterone. The mixture in syringe A was allowed to age for at least 15 min to allow the binding of the different components. The solution in syringe B was CO saturated prior to loading into the driving syringe.

The reaction was monitored via the CO complex formation showing an absorbance maximum at 450 nm and representing the formation of the reduced species. In total 6001 data points were recorded with a split time of 10/50 s. The dead time for the measurements with the SX-17MV was 2.6 ms. For each combination of redox partners three to four replicates traces were averaged. All resulting curves were evaluated using Origin. The reduction of CYP106A2 by ferredoxin species Adx(4–108) or Etp1(516–618) is best described by monoexponential relaxations, which were fitted to the data resulting in the apparent rate constants (k_{app}).

Data availability. The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request. The following previously published protein structures were used in this study: bovine Adx (PDB entry code 1AYF, chain B); adrenodoxin-like Etp1 (PDB entry code 2WLB, chain B); and CYP106A2 from *Bacillus megaterium* in substrate-bound form (PDB entry code 5IKI, chain B).

Received: 20 December 2017 Accepted: 27 June 2018

Published online: 30 July 2018

References

- Berg, A., Carlström, K., Gustafsson, J.-Å. & Ingelman-Sundberg, M. Demonstration of a cytochrome P-450-dependent steroid 15 β -hydroxylase in *Bacillus megaterium*. *Biochem. Biophys. Res. Commun.* **66**, 1414–1423 (1975).
- Berg, A., Gustafsson, J.-Å. & Ingelman-Sundberg, M. Characterization of a cytochrome P-450-dependent steroid hydroxylase system present in *Bacillus megaterium*. *J. Biol. Chem.* **251**, 2831–2838 (1976).
- Berg, A. & Rafter, J. J. Studies on the substrate specificity and inducibility of cytochrome P-450meg. *Biochem. J.* **196**, 781–786 (1981).
- Lisurek, M., Simgen, B., Antes, I. & Bernhardt, R. Theoretical and experimental evaluation of a CYP106A2 low homology model and production of mutants with changed activity and selectivity of hydroxylation. *Chembiochem* **9**, 1439–1449 (2008).
- Simgen, B., Contzen, J., Schwarzer, R., Bernhardt, R. & Jung, C. Substrate binding to 15 β -hydroxylase (CYP106A2) probed by FT infrared spectroscopic studies of the iron ligand CO stretch vibration. *Biochem. Biophys. Res. Commun.* **269**, 737–742 (2000).
- Bleif, S. et al. Identification of CYP106A2 as a regioselective allylic bacterial diterpene hydroxylase. *Chembiochem* **12**, 576–582 (2011).
- Schmitz, D., Zapp, J. & Bernhardt, R. Hydroxylation of the triterpenoid diptercarpol with CYP106A2 from *Bacillus megaterium*. *FEBS J.* **279**, 1663–1674 (2012).
- Bernhardt, R. Cytochromes P450 as versatile biocatalysts. *J. Biotechnol.* **124**, 128–145 (2006).
- Janocha, S., Schmitz, D., & Bernhardt, R. in *Biotechnology of Isoprenoids* (eds J. Schrader & J. Bohlmann.) 215–250 (Springer: Berlin, 2015).
- Schmitz, D., Janocha, S., Kiss, F. M. & Bernhardt, R. CYP106A2—a versatile biocatalyst with high potential for biotechnological production of selectively hydroxylated steroid and terpenoid compounds. *Biochim. Biophys. Acta (BBA)* **1866**, 11–22 (2018).
- Hannemann, F., Bichet, A., Ewen, K. M. & Bernhardt, R. Cytochrome P450 systems—biological variations of electron transport chains. *Biochim Biophys (BBA) Acta-Gen Subj.* **1770**, <https://doi.org/10.1016/j.bbagen.2006.07.017> (2007).
- Berg, A. Characterization of the ferredoxin component of the steroid 15 β -hydroxylase system from *Bacillus megaterium*. *Biochem. Biophys. Res. Commun.* **105**, 303–311 (1982).
- Berg, A., Ingelman-Sundberg, M. & Gustafsson, J.-Å. Purification and characterization of cytochrome P-450. *J. Biol. Chem.* **254**, 5264–5271 (1979).
- Berg, A., Ingelman-Sundberg, M. & Gustafsson, J.-Å. Isolation and characterization of cytochrome P-450meg. *Acta Biol. Med. Ger.* **38**, 333–344 (1978).
- Lisurek, M., Kang, M.-J., Hartmann, R. W. & Bernhardt, R. Identification of monohydroxy progesterones produced by CYP106A2 using comparative HPLC and electrospray ionisation collision-induced dissociation mass spectrometry. *Biochem. Biophys. Res. Commun.* **319**, 677–682 (2004).
- Ewen, K. M., Kleser, M. & Bernhardt, R. Adrenodoxin: the archetype of vertebrate-type [2Fe-2S] cluster ferredoxins. *Biochim. Biophys. Acta (BBA)-Proteins Proteom.* **1814**, 111–125 (2011).
- Brill, E. et al. A new cytochrome P450 system from *Bacillus megaterium* DSM319 for the hydroxylation of 11-keto- β -boswellic acid (KBA). *Appl. Microbiol. Biotechnol.* **98**, 1703–1717 (2014).
- Virus, C., Lisurek, M., Simgen, B., Hannemann, F. & Bernhardt, R. Function and engineering of the 15 β -hydroxylase CYP106A2. *Biochem. Soc. Trans.* **34**, 1215–1218 (2006).
- Bernhardt, R. & Waterman, M. R. Cytochrome P450 and steroid hormone biosynthesis. In *The Ubiquitous Roles Cytochrome P450 Proteins*, Vol. 3 (eds Sigel, A., Sigel, H. & Sigel, R. K. O.) 361–396 (Chichester, West Sussex, England, 2007).
- Uhlmann, H., Kraft, R. & Bernhardt, R. C-terminal region of adrenodoxin affects its structural integrity and determines differences in its electron transfer function to cytochrome P-450. *J. Biol. Chem.* **269**, 22557–22564 (1994).
- Muller, A. et al. New aspects of electron transfer revealed by the crystal structure of a truncated bovine adrenodoxin, Adx(4–108). *Structure* **6**, 269–280 (1998).
- Schiffler, B. et al. The interaction of bovine adrenodoxin with CYP11A1 (cytochrome P450sc) and CYP11B1 (cytochrome P45011 β) acceleration of reduction and substrate conversion by site-directed mutagenesis of adrenodoxin. *J. Biol. Chem.* **276**, 36225–36232 (2001).
- Bleif, S. et al. A new *Bacillus megaterium* whole-cell catalyst for the hydroxylation of the pentacyclic triterpene 11-keto- β -boswellic acid (KBA) based on a recombinant cytochrome P450 system. *Appl. Microbiol. Biotechnol.* **93**, 1135–1146 (2012).
- Milhim, M., Gerber, A., Neunzig, J., Hannemann, F. & Bernhardt, R. A novel NADPH-dependent flavoprotein reductase from *Bacillus megaterium* acts as an efficient cytochrome P450 reductase. *J. Biotechnol.* **231**, 83–94 (2016).
- Kleser, M., Hannemann, F., Hutter, M., Zapp, J. & Bernhardt, R. CYP105A1 mediated 3-hydroxylation of glimepiride and glibenclamide using a recombinant *Bacillus megaterium* whole-cell catalyst. *J. Biotechnol.* **157**, 405–412 (2012).
- Girhard, M., Klaus, T., Khatri, Y., Bernhardt, R. & Urlacher, V. B. Characterization of the versatile monooxygenase CYP109B1 from *Bacillus subtilis*. *Appl. Microbiol. Biotechnol.* **87**, <https://doi.org/10.1007/s00253-010-2472-z> (2010).
- Bakkes, P. J. et al. Design and improvement of artificial redox modules by molecular fusion of flavodoxin and flavodoxin reductase from *Escherichia coli*. *Scientific Rep.* **5**, 12158 (2015).
- Litzenburger, M., Kern, F., Khatri, Y. & Bernhardt, R. Conversions of tricyclic antidepressants and antipsychotics with selected P450s from *Sorangium cellulosum* So ce56. *Drug Metab. Dispos.* **43**, 392–399 (2015).
- Ringle, M., Khatri, Y., Zapp, J., Hannemann, F. & Bernhardt, R. Application of a new versatile electron transfer system for cytochrome P450-based *Escherichia coli* whole-cell bioconversions. *Appl. Microbiol. Biotechnol.* **97**, <https://doi.org/10.1007/s00253-012-4612-0> (2013).
- Schiffler, L. et al. Metabolism of oral turinabol by human steroid hormone-synthesizing cytochrome P450 enzymes. *Drug Metab. Dispos.* **44**, 227–237 (2016).
- Brixius-Anderko, S., Hannemann, F., Ringle, M., Khatri, Y. & Bernhardt, R. An indole-deficient *Escherichia coli* strain improves screening of cytochromes P450 for biotechnological applications. *Biotechnol. Appl. Biochem.* **64**, 315–326 (2017).
- Kang, M. J., Lisurek, M., Bernhardt, R. & Hartmann, R. W. Use of high-performance liquid chromatography/electrospray ionization collision-induced dissociation mass spectrometry for structural identification of monohydroxylated progesterones. *Rapid Commun. Mass Spectrom.* **18**, 2795–2800 (2004).
- Ewen, K. M., Schiffler, B., Uhlmann-Schiffler, H., Bernhardt, R. & Hannemann, F. The endogenous adrenodoxin reductase-like flavoprotein arh1 supports heterologous cytochrome P450-dependent substrate conversions in *Schizosaccharomyces pombe*. *FEMS Yeast Res.* **8**, <https://doi.org/10.1111/j.1567-1364.2008.00360.x> (2008).
- Bureik, M. et al. Development of test systems for the discovery of selective human aldosterone synthase (CYP11B2) and 11 β -hydroxylase (CYP11B1) inhibitors: discovery of a new lead compound for the therapy of congestive heart failure, myocardial fibrosis and hypertension. *Mol. Cell. Endocrinol.* **217**, 249–254 (2004).

35. Hobler, A. et al. Human aldosterone synthase: recombinant expression in *E. coli* and purification enables a detailed biochemical analysis of the protein on the molecular level. *J. Steroid Biochem. Mol. Biol.* **132**, 57–65 (2012).
36. Lawson, R. J., von Wachenfeldt, C., Haq, I., Perkins, J. & Munro, A. W. Expression and characterization of the two flavodoxin proteins of *Bacillus subtilis*, YkuN and YkuP: biophysical properties and interactions with cytochrome P450. *Biochemistry* **43**, 12390–12409 (2004).
37. Müller, J. J. et al. Structural and thermodynamic characterization of the adrenodoxin-like domain of the electron-transfer protein Etp1 from *Schizosaccharomyces pombe*. *J. Inorg. Biochem.* **105**, 957–965 (2011).
38. Uhlmann, H., Iametti, S., Vecchio, G., Bonomi, F. & Bernhardt, R. Pro108 is important for folding and stabilization of adrenal ferredoxin, but does not influence the functional properties of the protein. *FEBS J.* **248**, 897–902 (1997).
39. Bernhardt, R. & Urlacher, V. B. Cytochromes P450 as promising catalysts for biotechnological application: chances and limitations. *Appl. Microbiol. Biotechnol.* **98**, 6185–6203 (2014).
40. Rauschenbach, R., Isernhagen, M., Noeske-Jungblut, C., Boidol, W. & Siewert, G. Cloning sequencing and expression of the gene for cytochrome P-450meg, the steroid-15 α -monooxygenase from *Bacillus megaterium* ATCC 13368. *Mol. General. Genet.* **241**, 170–176 (1993).
41. Virus, C. & Bernhardt, R. Molecular evolution of a steroid hydroxylating cytochrome P450 using a versatile steroid detection system for screening. *Lipids* **43**, 1133–1141 (2008).
42. Janocha, S., Carius, Y., Hutter, M., Lancaster, C. R. & Bernhardt, R. Crystal structure of CYP106A2 in substrate-free and substrate-bound form. *ChemBiochem* **17**, 852–860 (2016).
43. Sambrook, J., Fritsch, E. F. & Maniatis, T. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).
44. Schiffler, B. et al. The adrenodoxin-like ferredoxin of *Schizosaccharomyces pombe* mitochondria. *J. Inorg. Biochem.* **98**, 1229–1237 (2004).
45. Bureik, M., Schiffler, B., Hiraoka, Y., Vogel, F. & Bernhardt, R. Functional expression of human mitochondrial CYP11B2 in fission yeast and identification of a new internal electron transfer protein, etp1 τ . *Biochemistry* **41**, <https://doi.org/10.1021/bi0157870> (2002).
46. Uhlmann, H., Beckert, V., Schwarz, D. & Bernhardt, R. Expression of bovine adrenodoxin in *E. coli* and site-directed mutagenesis of [2Fe-2S] cluster ligands. *Biochem. Biophys. Res. Commun.* **188**, 1131–1138 (1992).
47. Sagara, Y. et al. Direct expression of adrenodoxin reductase in *Escherichia coli* and the functional characterization. *Biol. Pharm. Bull.* **16**, 627–630 (1993).
48. Khatri, Y., Schiffrin, A. & Bernhardt, R. Investigating the effect of available redox protein ratios for the conversion of a steroid by a myxobacterial CYP260A1. *FEBS Lett.* **591**, 1126–1140 (2017).
49. Kozakov, D. et al. How good is automated protein docking? *Proteins* **81**, 2159–2166 (2013).
50. Kozakov, D., Brenke, R., Comeau, S. R. & Vajda, S. PIPER: an FFT-based protein docking program with pairwise potentials. *Proteins* **65**, 392–406 (2006).
51. Comeau, S. R., Gatchell, D. W., Vajda, S. & Camacho, C. J. ClusPro: a fully automated algorithm for protein–protein docking. *Nucleic Acids Res.* **32**, W96–W99 (2004).
52. Comeau, S. R., Gatchell, D. W., Vajda, S. & Camacho, C. J. ClusPro: an automated docking and discrimination method for the prediction of protein complexes. *Bioinformatics* **20**, 45–50 (2004).
53. Humphrey, W., Dalke, A. & Schulten, K. VMD: visual molecular dynamics. *J. Mol. Graphics* **14**, 33–38 (1996).
54. Pronk, S. et al. GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation toolkit. *Bioinformatics* <https://doi.org/10.1093/bioinformatics/btu055> (2013).
55. Jorgensen, W. L. & Madura, J. D., Quantum and statistical mechanical studies of liquids. 25. Solvation and conformation of methanol in water. *J. Am. Chem. Soc.* **105**, 1407–1413 (1983).
56. Berendsen, H. J., Postma, J., van Gunsteren, W. F., DiNola, A. & Haak, J. Molecular dynamics with coupling to an external bath. *J. Chem. Phys.* **81**, 3684–3690 (1984).
57. Parrinello, M. & Rahman, A. Polymorphic transitions in single crystals: a new molecular dynamics method. *J. Appl. Phys.* **52**, 7182–7190 (1981).
58. Hess, B. P-LINCS: A parallel linear constraint solver for molecular simulation. *J. Chem. Theory Comput.* **4**, 116–122 (2008).
59. Zöllner, A., Hannemann, F., Lisurek, M. & Bernhardt, R. Deletions in the loop surrounding the iron-sulfur cluster of adrenodoxin severely affect the interactions with its native redox partners adrenodoxin reductase and cytochrome P450 scc (CYP11A1). *J. Inorg. Biochem.* **91**, 644–654 (2002).

Acknowledgements

This work was supported by the German Federal Ministry of Education and Research (BMBF), grant FKZ 031A166A to R.B. and M.C.H. We are also thankful to Jens Neunzig and Frank Hannemann for providing their assistance with the stopped-flow measurements.

Author contributions

T.S. conceived and conducted most of the experiments, analyzed and interpreted the data associated with the experiments, designed the artwork, and is the main author of the manuscript. J.L.R. and M.C.H. conducted and evaluated MD simulation experiments. M.M. contributed to cloning, expression, and evaluation of the mutant. M.C.H. and R.B. designed the experiments and contributed to conceiving experiments, interpretation of data and writing of the manuscript. All authors reviewed and gave approval to the final version of the manuscript.

Additional information

Competing interests: The authors declare no competing interests.

Reprints and permission information is available online at <http://npg.nature.com/reprintsandpermissions/>

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2018

2.2 Sagadin et al. (2019)

Novel approach to improve progesterone hydroxylation selectivity by CYP106A2 via rational design of adrenodoxin binding

Tanja Sagadin, Jan Riehm, Natalia Putkaradze, Michael C. Hutter, Rita Bernhardt

The FEBS Journal 286 (2019) 1240-1249, doi: 10.1111/febs.14722



Novel approach to improve progesterone hydroxylation selectivity by CYP106A2 via rational design of adrenodoxin binding

Tanja Sagadin¹, Jan Riehm², Natalia Putkaradze¹, Michael C. Hutter² and Rita Bernhardt¹

¹ Department of Biochemistry, Saarland University, Saarbrücken, Germany

² Center for Bioinformatics, Saarland University, Saarbrücken, Germany

Keywords

Adrenodoxin; CYP106A2; cytochrome P450; electron transfer; molecular dynamics simulation

Correspondence

R. Bernhardt, Department of Biochemistry, Saarland University, Campus B2.2, 66123 Saarbrücken, Germany
Fax: +49 681 302 4739
Tel: +49 681 302 4241
E-mail: ritabern@mx.uni-saarland.de and

M. C. Hutter, Center for Bioinformatics, Saarland University, Campus E2.1, 66123 Saarbrücken, Germany
Fax: +49 681 302 70702
Tel: +49 681 302 70703
E-mail: michael.hutter@bioinformatik.uni-saarland.de

(Received 9 July 2018, revised 9 November 2018, accepted 3 December 2018)

doi:10.1111/febs.14722

Bacterial P450s have considerable potential for biotechnological applications. The P450 CYP106A2 from *Bacillus megaterium* ATCC 13368 converts progesterone to several hydroxylated products that are important precursors for pharmaceutical substances. As high yields of monohydroxylated products are required for biotechnological processes, improving this conversion is of considerable interest. It has previously been shown that the binding mode of the redox partner can affect the selectivity of the progesterone hydroxylation, being more stringent in case of the Etp1 compared with Adx(4–108). Therefore, in this study we aimed to improve hydroxylation selectivity by optimizing the binding of Adx(4–108) with CYP106A2 allowing for a shorter distance between both redox centers. To change the putative binding interface of Adx(4–108) with CYP106A2, molecular docking was used to choose mutation sites for alteration. Mutants at positions Y82 and P108 of Adx were produced and investigated, and confirmed our hypothesis. Protein–protein docking, as well as conversion studies, using the mutants demonstrated that the iron–sulfur (FeS) cluster/heme distance diminished significantly, which subsequently led to an approximately 2.5-fold increase in 15 β -hydroxyprogesterone, the main product of progesterone conversion by CYP106A2.

Introduction

Besides their evolutionary role in the biosynthesis of endogenous substances, cytochrome P450 enzymes are also capable of converting exogenous chemicals [1]. Particularly bacterial and fungal cytochromes P450 are interesting for biotechnological applications, especially the biosynthesis of otherwise difficult to obtain products [2]. For example, CYP106A2 from *Bacillus megaterium* ATCC 13368 is able to hydroxylate various 3-oxo- Δ 4-steroids (e.g., progesterone, prednisone, dexamethasone,

androstenedione) predominately at the 15 β position [3]. This formal oxygen insertion into a nonactivated C–H bond is extremely difficult by means of conventional chemical synthesis, but corresponding products and their derivatives can be used as pharmaceutical substances or as precursors for further synthetic steps toward steroid-like compounds [4,5]. Terpenoids make CYP106A2 very attractive for applications in biotechnology [4,6]. The availability of the high-resolution

Abbreviations

Adx(4–108), shortened adrenodoxin comprising residues 4–108; Adx, adrenodoxin; Etp1(516–618), electron transfer protein 1 comprising residues 516–618.

crystallographic structure of CYP106A2 [7] does not only allow the application of molecular docking studies to assist the interpretation of product spectra but, furthermore, enables the rational design of mutants that show altered product distributions [8]. Likewise, the choice of the redox partner – the ferredoxin, which delivers the electrons – influences the amount and kind of products being formed. As shown before, the enzymatic conversion of progesterone using bovine adrenodoxin Adx(4–108) as an electron donor for CYP106A2 produces, besides the main product 15 β -hydroxyprogesterone (15 β -OH-P), several monohydroxylated side products as well as polyhydroxylated progesterone [8–10]. In contrast, the adrenodoxin-like electron transfer protein 1 [Etp1(516–618)] from *Schizosaccharomyces pombe*, which is homologous both in sequence and structure [11] to bovine adrenodoxin, surprisingly was found to produce a substantially lower amount of side products [10].

Etp1(516–618) exhibits 81% sequence identity to the truncated, yet fully functional Adx(4–108), and is also highly similar in its three-dimensional structure [11]. Particularly in the regions around the FeS cluster, any substantial deviations of the protein backbone are absent. One may, therefore, speculate that this highly conserved region is functionally important, either for the binding of the FeS cluster or as part of the putative protein–protein interface or electron transfer pathway. Multiple sequence alignment with further closely related ferredoxins (e.g., from *Escherichia coli*, *Rhodobacter capsulatus*, *Pseudomonas putida*, and *Saccharomyces cerevisiae*) also possessing the Fe₂S₂ cofactor, however, shows that amino acids are only identical in the very close vicinity of the four cysteines that ligate the two iron atoms, whereas other regions can vary both in amino acid content and structure (Fig. 1).

The above mentioned differences seen in the product spectra upon the conversion of progesterone by Adx(4–108) and Etp1(516–618) as electron donors to CYP106A2 were thus explained by subtle changes in amino acids between Etp1 and Adx that lead to different binding modes to CYP106A2 [10]. The results from protein–protein docking studies showed a substantial increase in the FeS cluster/heme distance when applying Adx(4–108) instead of Etp1(516–618). Thus, binding of Etp1 is facilitated, which leads to faster electron transfer. This structural background, linking ferredoxin structure and binding to product spectra, gave hints that it should be possible, based on structural data and molecular docking, to improve the binding of Adx(4–108) to CYP106A2 by mutating the concerned amino acids and tune this way the

functionality of Adx(4–108). Therefore, we replaced amino acids of Adx(4–108) to those present in Etp1(516–618) to improve binding of the mutant to CYP106A2 and this way the selectivity of hydroxylation. Our data demonstrated that mutating the ferredoxin component represents a novel approach to design P450 systems with altered selectivities regarding product patterns.

Results

Selectivity and product distribution of steroid conversions by bacterial P450s is not only dependent on properties of amino acids in the active site of the protein but also dependent on the redox partners, which can drastically influence the amount of polyhydroxylated products being formed [10]. Obviously, spacial variations in the used ferredoxins, that is, Etp1(516–618) and Adx(4–108), influence binding, and thus electron transfer, to the P450. As shown by molecular docking investigations [10], certain amino acids of Adx(4–108) prevent it from binding to CYP106A2 as closely as Etp1(516–618). From this observation the question arose whether mutations of the relevant amino acids in Adx(4–108) that make this region more similar to Etp1(516–618), will change the selectivity of progesterone conversion by CYP106A2 toward a higher yield of the main product, 15 β -OH-progesterone.

Protein–protein docking studies of Adx(4–108) and Etp1(516–618) to CYP106A2 were performed and putative sites for replacement of amino residues in the region around the binding interface were deduced. The molecular centers of the redox system are the FeS cluster contained in the ferredoxins [either Adx(4–108) or Etp1(516–618)], and the heme group of the CYP106A2. Both contain iron, which can undergo a change in its formal oxidation state. Although the Fe₂S₂ cofactor of the FeS cluster is ligated by four cysteine residues and is located near to the surface of the ferredoxins, the heme group is more buried within CYP106A2 as seen in the X-ray structure (pdb entry 5IKI)[7] (Fig. 2). Thus, electron transfer is unlikely to proceed directly from the FeS cluster to the heme iron. Instead, the phenylalanine residues 107, 348, and 354 of CYP106A2 are found close to the heme group and may function as relays, whereby Phe107 and Phe354 are solvent exposed. Therefore, we assume that the region around these residues is the putative contact surface between the ferredoxins and CYP106A2.

From the structural comparison of the complex between the homologous human Adx and CYP11A1 as given in the crystallographic structure (pdb entry 3N9Y) [12] we hypothesized that the side chain of

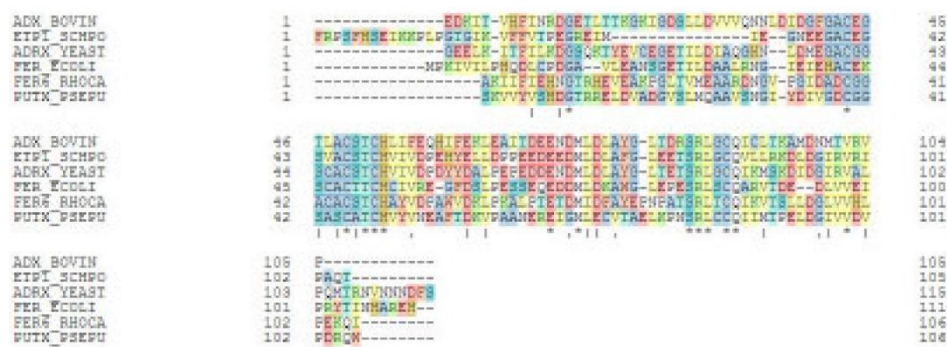


Fig. 1. Multiple sequence alignment of the ferredoxins Adx (bovine), Etp1 (*Schizosaccharomyces pombe*), Adrx (*Saccharomyces cerevisiae*), Fdx (*Escherichia coli*), fdxE (*Rhodobacter capsulatus*), and putidaredoxin (*Pseudomonas putida*). Amino acids are shaded according to their degree of conservation. Highly conserved ones are marked with asterisks, whereas exchanges by similar amino acids are denoted by colons or periods, respectively.

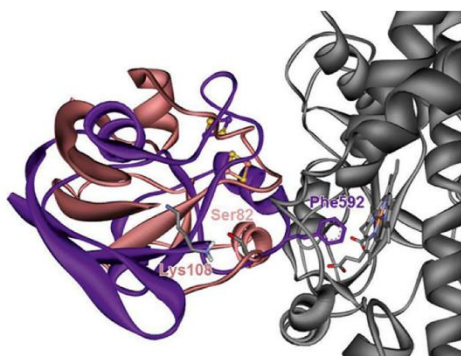


Fig. 2. Overlaying the obtained docking conformation of Adx(4-108)Y82S/P108K (coral) and that of Etp1(516-618) (violet) to CYP106A2 (gray) shows that the FeS cluster of the Adx mutant is closer to the heme group of CYP106A2 than that of Etp1 (516-618). Lys108 and Ser82 of Adx form an intermolecular hydrogen bond, whereas the corresponding phenylalanine of Etp1(516-618) deeply penetrates into CYP106A2. Iron atoms of the FeS clusters are shown in the color of the respective ferredoxin.

residue 82 of Adx is part of the contact interface with CYP106A2. Adx mutants were previously constructed in our lab that had Tyr82 replaced by phenylalanine, leucine, and serine [13]. These mutants did not show substantial changes compared to wild-type Adx of their $V_{\max}$ values of CYP11A1- and CYP11B1-dependent activities, but showed variations in K_m leading to the suggestion that mutants of Y82 directly or indirectly (by inducing small conformational changes)

affect binding of Adx to cytochromes P450. Further considerations regarding the choice of mutations came from our previous results of protein–protein docking of Etp1(516–618) to CYP106A2 [10]. For Etp1, we found that the side chain of phenylalanine that corresponds to Tyr82 in adrenodoxin deeply penetrates into CYP106A2 by forming hydrophobic contacts with Phe107, Pro359, and Leu356. Conversely, no hydrogen-bonding partner for Tyr82 of Adx(4–108) is present in CYP106A2 and, thus, the corresponding binding position would be disfavored energetically. Moreover, subtle structural differences in Adx(4–108) prevent it from adopting a binding to CYP106A2 as it was found for Etp1(516–618), because residues around positions 14 and 41 would sterically clash with CYP106A2. Instead of altering these regions, we conceived mutants of Adx(4–108) where Pro108 was replaced by either arginine or lysine. In combination with corresponding mutations of Tyr82 into serine or aspartate, these introduce intermolecular hydrogen bonds or salt bridges, respectively. As a consequence, we expected that the side chain of residue 82 is drawn toward the surface of Adx and thus exhibits less steric hindrance upon binding to CYP106A2. This should then lead to the expected change in the selectivity of progesterone hydroxylation.

To get further insight into the binding interface between cytochromes and ferredoxins, we compared the recognition surface of several complexes for which experimental structural information is available with those obtained from protein–protein docking. The X-ray structure of the of complex between CYP11A1 and bovine Adx (3N9Y.pdb) shows only

the residues 40–90 of the ferredoxin, which are in close vicinity to the cytochrome. For protein–protein docking we, therefore, used the structure given by 1AYF.pdb (chain B), which comprises residues 5–108. To allow comparison to the docked conformation, this structure was superimposed onto that of Adx in the complex. Although the RMSD (backbone atoms) of the predicted conformation of Adx is 5.2 Å, the orientation of the actual binding interface is fully reproduced. Considering residues 40–90 of the Adx present in the crystal structure, the RMSD reduces to 3.4 Å.

For the related P450–ferredoxin complexes of CYP199A2–HaPux (top ranked complex from structure determination by orientation-selective double electron–electron) [14] and of CYP101A1–Pdx (pdb entry 4JWS.pdb) [15] the obtained RMSD values are much higher (14.1 Å and 13.6 Å, respectively). Visual inspection, however, showed that the binding site is matched, but the ferredoxins are somewhat skewed. It should be kept in mind that RMSD values are more sensitive to rotational deviations than to translations. In this binding region, CYP101A2 and CYP199A2 are structurally more similar to each other than to CYP106A2. In contrast to the former P450s, it possesses a short α -helical segment comprising residues Asn342, Lys343, and Lys344, whereas charged amino acids are absent in the corresponding positions of the two other P450s.

Interestingly, both lysine side chains are predicted to form extensive electrostatic interactions with Adx(4–108) via the oxygen atoms of Glu47 and of Thr49.

The results from protein–protein docking using the suggested mutants of Adx(4–108) showed that all of these mutants displayed even shorter distances between the FeS cluster and the heme iron than it was found for Etp1(516–618) (Table 1). Furthermore, the balanced docking score obtained from the protein–protein docking revealed important differences between the ‘parent’ Adx(4–108) and the suggested Adx(4–108) mutants. Thus, the modified residues should allow a different binding position at CYP106A2. This becomes apparent when overlaying the obtained binding positions of the Adx(4–108)Y82S/P108K mutant and that of Etp1(516–618) (Fig. 2).

The corresponding Adx(4–108) mutants were constructed by site-directed mutagenesis, expressed at 37 °C, and purified as described for Adx(4–108) [16]. The mutants were less stable than Adx(4–108) and, thus, were not purified via the known protocol. However, by lowering the expression temperature to 30 °C, the mutants were obtained as stable proteins.

The change in selectivity was verified in a reconstituted *in vitro* assay for the conversion of progesterone

Table 1. Calculated distances between the redox centers of the ferredoxins and CYP106A2 obtained from protein–protein docking and ClusPro2 score. The minimal distance found in the five most populated conformation clusters of each mutant is given.

Ferredoxin	Calculated minimal distance between the FeS cluster and heme iron	ClusPro2 score ^a
Adx(4–108)	24.1 Å	–613.7
Adx(4–108)P108R	11.7 Å	–599.9
Adx(4–108)Y82S/P108K	13.4 Å	–509.9
Adx(4–108)Y82S/P108R	13.2 Å	–539.5
Adx(4–108)Y82D/P108K	11.1 Å	–509.2
Adx(4–108)Y82D/P108R	14.3 Å	–550.2
Etp1(516–618)	19.1 Å	–673.7

^aBalanced docking score for the obtained docking model that is the representative center of the most populated cluster.

with CYP106A2. All mutants were able to convert 200 μ M of progesterone within 60 min, just as Adx(4–108). As expected, compared with Adx(4–108), the mutants produced much more of the main product (15 β -OH-P) after 60 min of conversion than their parent enzyme (Table 2).

In vitro conversions were also conducted in time intervals from 1 min up to 60 min to elucidate the time dependency of the product formation. All mutants of Adx(4–108) produced more main product (15 β -OH-progesterone) within 1 h than the parent Adx(4–108) (Fig. 3, shown here for mutant Adx(4–108)Y83S/P108R). Looking at the time course of the measurements, it becomes obvious that despite the fast conversion of progesterone with all tested ferredoxins, which is almost complete within 5 min, the product distribution changes drastically. Using Adx(4–108), the amount of the main product decreases as 15 β -OH-P becomes polyhydroxylated. In contrast, if Etp1(516–

Table 2. Experimentally observed product ratios of hydroxylated progesterone after 60 min of conversion. Fractions of products are given with standard deviation. Three conversions were conducted independently.

Ferredoxin	Fraction of	
	15 β -OH-progesterone	Poly-OH-progesterone
Adx(4–108)	35% $\pm$ 0.050	59% $\pm$ 0.047
Adx(4–108)P108R	67% $\pm$ 0.059	20% $\pm$ 0.068
Adx(4–108)Y82S/P108K	74% $\pm$ 0.008	16% $\pm$ 0.008
Adx(4–108)Y82S/P108R	71% $\pm$ 0.012	6% $\pm$ 0.014
Adx(4–108)Y82D/P108K	68% $\pm$ 0.040	18% $\pm$ 0.019
Adx(4–108)Y82D/P108R	72% $\pm$ 0.072	19% $\pm$ 0.065
Etp1(516–618)	81% $\pm$ 0	14% $\pm$ 0

618) or one of the mutants of Adx(4–108) is used, the main product remains on the same level, whereas the polyhydroxylated products appear only in small quantities over the time course of 60 min.

To investigate whether, besides the structural fit of both proteins, the redox potential could play a role in modulating the selectivity of the analyzed progesterone hydroxylation, we measured the redox potential of the obtained mutants. As seen in Table 3, mostly very small or no changes between parent Adx(4–108) and the mutants were found. Also, no correlation between the redox potential and the yield of the main product as shown in Table 2 is recognizable. Thus, it can be stated that the redox potential does not influence the selectivity of hydroxylation catalyzed by CYP106A2.

Furthermore, we determined the coupling efficiency of the three systems shown in Fig. 3. This experiment revealed different coupling efficiencies when using Adx(4–108), Etp1(516–618), or the Adx mutants (Fig. 4).

Discussion

Ferredoxins as electron transfer partners to P450s are possible points of intervention when looking for optimization of these redox systems. It was previously shown in our laboratory that the change in redox partners caused different product patterns *in vitro*, as seen with CYP106A2 and progesterone as substrate [10]. Moreover, the use of Fpr (*E. coli*) instead of AdR led to higher turnover numbers for hydroxylations of steroids *in vivo* with different P450s [17]. The applied ratios of the redox system components (P450, ferredoxin, ferredoxin reductase) likewise exhibited substantial effects on the steroid product pattern of the myxobacterial CYP260A1 [18]. Furthermore, it was reported recently that fusions of P450s with compatible redox systems displayed physiological reactions, whereas free enzymes showed non-natural ones [19]. These different approaches allow the modulation of the conversion of substrates by P450s via altered or optimized redox partners. In this study, we aimed to change the selectivity of the progesterone conversion by mutating amino acids of Adx(4–108) which hinder the close binding to CYP106A2. Considering previous studies of our laboratory concerning the effects of mutations of Adx [20–22] on P450 binding, as well as molecular docking of Adx(4–108) to CYP106A2, two positions (amino acids 82 and 108) were chosen for mutations. Due to our computer modeling studies, introduction of a smaller residue at position 82 and the replacement at position 108 should lead to a shorter distance between the FeS cluster and the heme, by allowing a closer approach of the two involved

cofactors. This shorter distance and tighter binding was expected to alter the selectivity of the progesterone conversion by CYP106A2 to that shown previously for Etp1(516–618) [10].

After the construction of the mutants, differences in the expression schedule were necessary for the Adx mutants, because at high expression temperatures only a low protein yield per liter culture volume was obtained, in accordance with previous observations [22]. The goal to change the selectivity of progesterone conversion was verified by *in vitro* conversion studies. First, the overall functionality of electron transport to the P450 was proven, as 200 μ M progesterone was converted completely by the mutants as well as the parent Adx(4–108) within 60 min. However, as expected and shown in Table 2, the fractions of the main product, 15 β -OH-P, and the unwanted, polyhydroxylated progesterone change substantially: after 60 min of conversion, the use of Adx(4–108) leads to 35% 15 β -OH-P and 59% poly-OH-P. In contrast, the mutants of Adx(4–108) form between 67% and 74% 15 β -OH-P, and only 6% to 20% poly-OH-P. This change in product pattern is similar to the one obtained when using Etp1(516–618). The time course measurements (Fig. 3) confirm these suggestions: all constructed mutants show a product pattern variation over the time of 60 min that is similar to that of Etp1(516–618) as shown here for Adx(4–108)Y82S/P108R; AdR as an exemplary example. Our results from protein–protein docking obtained with ClusPro2 [ClusPro2] confirmed our assumptions regarding the consequences of the intermolecular contacts between the mutated residues in position 82 and 108. The respective amino acid side chains in position 82 (serine or aspartate, respectively) fold toward the protein surface to form polar contacts to the residues at position 108 (arginine or lysine, respectively) and, therefore, remove steric hindrance as well as the need for hydrogen bonding with residues of CYP106A2. All corresponding Adx(4–108) mutants (P108R, Y82S/P108K, Y82S/P108R, Y82D/P108K, and Y82D/P108R) were found in docking conformations that had the FeS cluster in closer distance than Adx(4–108) (Table 1). Even more, we were able to obtain results from computational calculations concerning the relationship between the fraction of 15 β -OH-progesterone being formed and the score obtained from protein–protein docking of the ferredoxins to the cytochrome. For the six Adx mutants Pearson's correlation coefficient is $r = 0.723$, indicating that stronger binding to the cytochrome leads to a larger fraction of 15 β hydroxylation. This also holds true for Etp1 which, however, shows a substantially stronger binding score as it differs much more from all other adrenodoxines.

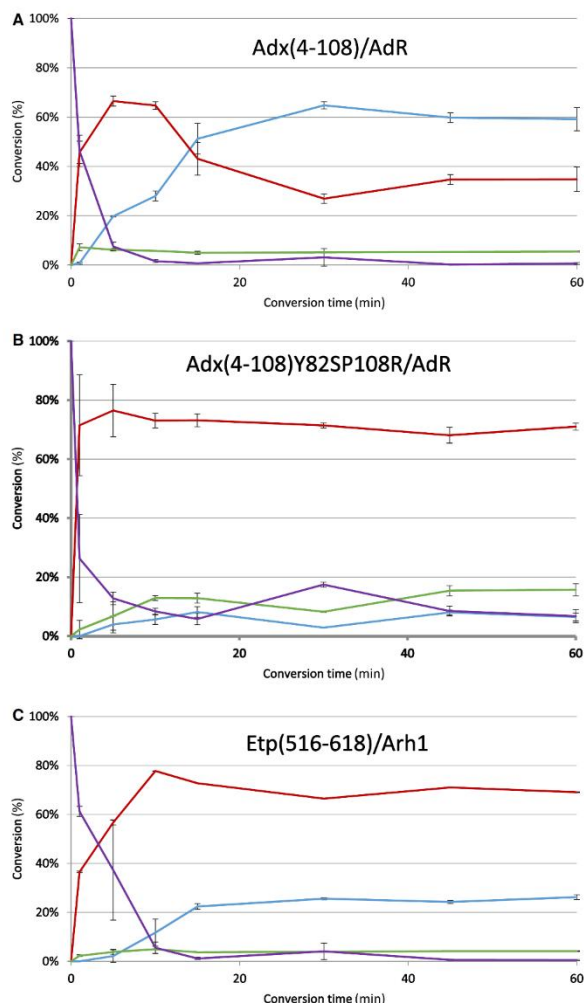


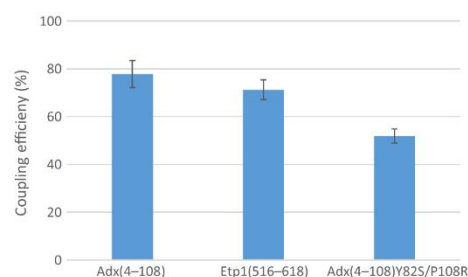
Fig. 3. Time-dependent *in vitro* conversion of progesterone by CYP106A2 and A: Adx(4–108);AdR, B: Adx(4–108)Y82SP108R;AdR, or C: Etp(516–618);Arh1. Conversions were stopped after 1, 5, 10, 15, 30, 45, or 60 min, respectively; blue lines indicate polyhydroxylated progesterone products, red lines indicate the main product 15β-OH-P, green lines indicate other monohydroxylated progesterone products, violet lines indicate the remaining substrate progesterone. Error bars show the standard deviation. The data represent average values of three independent reactions.

Since electrostatic contributions are the dominant term in the balanced docking score [23–25], we assume that the binding of the ferredoxins to CYP106A2 is governed by these forces. Interestingly, the intermolecular interaction between residues 108 and 82 also seems to stabilize the Adx(4–108) structure itself. Previous studies in our lab showed that removing proline in position 108 leads to a hole in the protein structure, which consequently facilitates its unfolding, because urea from the solvent can now penetrate more easily [22].

The experimentally observed hydroxylation pattern of progesterone, in particular the formation of polyhydroxylated products, requires the reorientation of monohydroxylated progesterone. Previously we were able to show by molecular dynamics simulations of 15β-OH-progesterone in CYP106A2 that the reorientation into conformations that allow further hydroxylation in other positions occurs within 100 ns [10]. Therefore, this process competes considerably with the product release from the binding pocket. We thus

Table 3. Parent and mutant Adx(4–108) and their measured redox potentials. The standard deviation of the redox potentials was obtained from three independent measurements.

Ferredoxins	Measured redox potential [mV]
Adx(4–108)	–325 ± 2
Adx(4–108)P108R	–319 ± 22
Adx(4–108)Y82S/P108K	–336 ± 12
Adx(4–108)Y82S/P108R	–314 ± 3
Adx(4–108)Y82D/P108K	–342 ± 24
Adx(4–108)Y82D/P108R	–373 ± 27

**Fig. 4.** Coupling efficiencies of the three systems Adx(4–108), Etp1(516–618), and Adx(4–108)Y82S/P108R. For determination of the NADPH coupling efficiency the *in vitro* reaction mixtures lacking G6P-dehydrogenase were used. The molar ratio of NADPH: progesterone was 1 : 1 (100 μ M each) and thus the coupling efficiency was calculated as the percentage of substrate converted by CYP106A2 over the consumed NADPH. Error bars show the standard deviation. The data represent average values of three independent reactions.

suggest that fast transfer of the two electrons favors the formation of the main hydroxylation product (15 β -OH-progesterone), whereas a longer dwell time in the binding pocket due to slower supply of electrons leads to increased formation of polyhydroxylated progesterone.

When combining the results from protein–protein docking with the experimental measurements, a consistent picture emerges. The speed, respectively the efficacy of electron transport by the investigated ferredoxins, seems to be governed by one important effect: the distance between the two redox centers (FeS cluster of the ferredoxin and the heme group of the P450). Shorter distances allow faster electron transfer, although we assume that there is no direct transfer between the redox centers as these are separated by several amino acids in any case. Thus, the obtained distances cannot be used to generate a linear dependence to the fraction of the main hydroxylation product. We also inspected the influence of the redox potentials of

the Adx(4–108) mutants. In most cases, the effect of the amino acid replacement was not significant. No correlation between the redox potential and the selectivity of progesterone hydroxylation could be drawn.

Taken together, our approach to improve the regioselectivity of progesterone hydroxylation by CYP106A2 via changing the redox partner Adx(4–108) to influence its binding to CYP106A2 creating a shorter distance between the two active sites of the proteins was very successful and paves the way for a novel approach to improve bacterial steroid hydroxylations.

Conclusions

In this study, we investigated the connection between electron donor structure, its binding to P450, and subsequently changed product pattern. Our goal was to increase the amount of the main product of CYP106A2-dependent progesterone conversion (15 β -OH-P) by mutations of the electron donor Adx(4–108) altering the binding mode to CYP106A2. The design aimed to obtain a minimal distance (similar to that between Etp1(516–618) and CYP106A2) between the heme iron of CYP106A2 and the FeS cluster of the given ferredoxin. By analyzing amino acids that mediate the interaction of Adx(4–108) or Etp1(516–618) to CYP106A2 we identified residues that lead to steric changes at the protein surface of these ferredoxins, and consequently result in different binding modes. As using Etp1(516–618) positively influences the product pattern of progesterone conversion towards a higher main product yield, we mutated Adx(4–108) accordingly to achieve a similar binding to CYP106A2. The mutations resulted in the favored improvement of the yield of the main product, bringing the Adx(4–108) mutants onto the same level as Etp1(516–618). With this strategy, we proved a new design possibility to tune the selectivity of a P450 steroid conversion by rational design of protein–protein interactions and electron transfer. The results are very important for the basic understanding of electron transport and its consequences in P450 conversions. They also have biotechnological importance as they can influence the economics of biotechnological applications by providing a higher yield of pharmaceutically or biotechnologically important products.

Methods

Site-directed mutagenesis

All Adx(4–108) mutants were constructed in the expression vector pKKHC. The primers used for the site-directed mutagenesis have been synthesized by Eurofins MWG

Operon and are listed in Table 4. The primers were used to construct the mutants Adx(4–108)P108R, Adx(4–108)Y82S/P108R, Adx(4–108)Y82D/P108R, Adx(4–108)Y82S/P108K, and Adx(4–108)Y82D/P108K by standard protocols [26], in one or two mutagenesis steps, for the single or double mutants, respectively.

Determination of redox potentials of Adx mutants

Determinations of the Adx(4–108) mutant redox potentials were executed using the dye photoreduction method [27] with safranin T as the redoxactive dye. Samples contained 10 μ M safranin T, 2% glucose, 35 μ L of an oxygen removing system (30 U glucose oxidase and 1300 U catalase), and 10 μ M of the individual Adx(4–108) mutant in a 50 mM potassium phosphate buffer containing 10 mM EDTA (ethylene diamine tetra-acetic acid) for proton supply. To remove the remaining oxygen, the mixture was aerated with argon in a rubber-sealed cell for 30 min in the dark and afterward kept in the dark under argon atmosphere for a further 30 min. The reduction of the dye was initiated by illumination of the sample with a 150 W lamp. Spectra were recorded in the range from 380 to 560 nm. Data were analyzed using the Nernst equation.

Protein expression in *Escherichia coli* and purification

Adx(4–108) mutants were expressed and purified as described previously [16] with an expression temperature of 30 °C. Also the elongation of the washing step in the purification procedure was an important feature to get to the purified stable proteins. CYP106A2 and Etp1(516–618) were expressed and purified as described elsewhere [28–32]. Adx(4–108) and AdR were provided by Birgit Heider-Lips and purified as described before [33,34].

In vitro conversion and HPLC analysis

Investigation of activity of adrenodoxin mutants was performed by CYP106A2-dependent progesterone *in vitro*

conversion assays and HPLC analysis as described previously [10]. To check the time dependency of the product formation *in vitro*, the reaction was stopped after 1, 5, 10, 15, 30, 45, and 60 min.

Computational methods

The homology models of the adrenodoxin mutants were constructed applying the corresponding functions of the Swiss-PdbViewer (<https://spdbv.vital-it.ch/>) [35] to the crystal structure of bovine adrenodoxin (pdb entry code 1AYF, chain B), which comprises residues 5–108 [23]. The adrenodoxin-like electron transfer protein 1 [Etp1(516–618)] was prepared likewise (pdb entry code 2WLB, chain B) [11]. As the receptor, the conformation of CYP106A2 from *B. megaterium* in substrate bound form was used (pdb entry code 5IK1, chain B) [7]. For protein–protein docking the ClusPro2 web service (<https://cluspro.org/login.php>) was used, applying default parameters [24,25,36,37]. Due to the limitations of the underlying method, all nonprotein parts [heme group of the P450 as well as the Fe₂S₂ fragment of adrenodoxin and of etp1(516–618)] were removed prior to the docking procedure. For further analysis, the complexes obtained according to the ‘balanced scoring function’ were used. These were inspected manually by superimposing the heme containing structure of CYP106A2 within vmd (version 1.8.6, <http://www.ks.uiuc.edu/Research/h/vmd/>) [38].

Molecular dynamics simulations were carried out on 15 β -OH-progesterone in the binding pocket of CYP106A2 applying GROMACS (version 4.4.5) [GROMACS]. The amino acids that were unresolved in the crystal structure were modeled using the Swiss-PdbViewer. The P450 was placed in cubic box with a minimum distance of 10 Å from the box edges and surrounded by TIP3P waters [39]. Energy minimization was started from the putative conformation of 15 β -OH-progesterone after hydroxylation. The steepest descent energy minimization was stopped at a gradient norm lower than 1000 kJ·mol^{−1}·nm^{−2}. Equilibration consisted in two different steps: first in a NVT (constant volume, temperature, and number of particles) ensemble for 100 ps maintaining the temperature at 300 K using a modified Berendsen

Table 4. Primers used to construct Adx(4–108) mutants at positions 82 and 108. The specific mutation is listed in the first column, followed by the given name of the used primer and the sequence of the primer (changed nucleotides are underlined).

Mutation	Primer name	Sequence
P108R (forward)	AdxForP108R	CTGTTTCGAGTACGCTAAGCTTGGC
P108R (reverse)	AdxRevP108R	GCCAAGCTTAGCTACTCGAACAG
P108K (forward)	AdxForP108K	GACTGTTTCGAGTAAAGTAAGCTTGGCTG
P108K (reverse)	AdxRevP108K	CAGCCAAGCTTACTTTACTCGAACAGTC
Y82S (forward)	AdxForY82S	GCTTGATCTGGCATCTGGACTAACAG
Y82S (reverse)	AdxRevY82S	CTGTTAGTCCAGATGCCAGATCAAGC
Y82D (forward)	AdxForY82D	GCTTGATCTGGCAGATGGACTAACAG
Y82D (reverse)	AdxRevY82D	CTGTTAGTCCATCTGCCAGATCAAGC

thermostat [40], and second by stabilizing the system at 1 bar applying a Parinello–Rahman barostat [41] for 100 ps of NPT (constant pressure, temperature, and number of particles) equilibration. All bonds in both steps were constrained by the LINCS algorithm [42]. Subsequently, the production run of 100 ns was performed. Hereby, Newton's equations of motion were integrated by the leapfrog algorithm at time steps of 2 fs. Snapshots of the trajectory were taken at intervals of 1 ps.

Coupling efficiency

For determination of the NADPH coupling efficiency the *in vitro* reaction mixtures lacking G6P-dehydrogenase were used. The mixtures were preheated at 30 °C and the reactions were started by the addition of NADPH solution. NADPH consumption was monitored at 339 nm and 30 °C by using a Jasco UV-Vis spectrophotometer V-630. After NADPH consumption the reactions were extracted by ethyl acetate and progesterone conversion was measured by HPLC (as described for *in vitro* reactions). The molar ratio of NADPH:progesterone was 1 : 1 (100 µM each) and thus the coupling efficiency was calculated as the percentage of substrate converted by CYP106A2 over the consumed NADPH. The data represent average values of three independent reactions.

Acknowledgements

This work was supported by the German Federal Ministry of Education and Research (BMBF), grant FKZ 031A166A. We thank Frank Hannemann for the help measuring the redox potentials.

Author contributions

TS conceived and conducted most of the experiments, analyzed and interpreted the data associated with the experiments, designed the artwork, and is the main author of the manuscript. JLR and MCH conducted and evaluated MD simulation experiments. NP conducted the coupling efficiency experiments. MCH and RB contributed to conceiving experiments, interpretation of data, and writing of the manuscript. All authors reviewed and gave approval to the final version of the manuscript.

References

- Bernhardt R (2006) Cytochromes P450 as versatile biocatalysts. *J Biotechnol* **124**, 128–145.
- Bernhardt R & Urlacher VB (2014) Cytochromes P450 as promising catalysts for biotechnological application: chances and limitations. *Appl Microbiol Biotechnol* **98**, 6185–6203.
- Berg A, Gustafsson J-A & Ingelman-Sundberg M (1976) Characterization of a cytochrome P-450-dependent steroid hydroxylase system present in *Bacillus megaterium*. *J Biol Chem* **251**, 2831–2838.
- Schmitz D, Janocha S, Kiss FM & Bernhardt R (2018) CYP106A2—a versatile biocatalyst with high potential for biotechnological production of selectively hydroxylated steroid and terpenoid compounds. *Biochim et Biophys Acta (BBA)-Prot Proteom* **1866**, 11–22.
- Donova MV & Egorova OV (2012) Microbial steroid transformations: current state and prospects. *Appl Microbiol Biotechnol* **94**, 1423–1447.
- Janocha S, Schmitz D & Bernhardt R (2015) Terpene hydroxylation with microbial cytochrome P450 monooxygenases. In *Biotechnology of Isoprenoids* (Schrader J & Bohlmann J, eds), vol 148, pp. 215–250. Springer, Cham.
- Janocha S, Carius Y, Hutter M, Lancaster CR & Bernhardt R (2016) Crystal structure of CYP106A2 in substrate-free and substrate-bound form. *ChemBioChem* **17**, 852–860.
- Nikolaus J, Nguyen KT, Virus C, Riehm JL, Hutter M & Bernhardt R (2017) Engineering of CYP106A2 for steroid 9 α - and 6 β -hydroxylation. *Steroids* **120**, 41–48.
- Nguyen KT, Virus C, Günnewich N, Hannemann F & Bernhardt R (2012) Changing the regioselectivity of a P450 from C15 to C11 hydroxylation of progesterone. *ChemBioChem* **13**, 1161–1166.
- Sagadin T, Riehm JB, Hutter MC & Bernhardt R (2018) Binding modes of CYP106A2 redox partners determine differences in progesterone hydroxylation product patterns. *Communicat Biol* **1**, 99.
- Müller JJ, Hannemann F, Schiffler B, Ewen KM, Kappl R, Heinemann U & Bernhardt R (2011) Structural and thermodynamic characterization of the adrenodoxin-like domain of the electron-transfer protein Etp1 from *Schizosaccharomyces pombe*. *J Inorg Biochem* **105**, 957–965.
- Strushkevich N, Mackenzie F, Cherkasova T, Grabovec I, Usanov S & Park HW (2011) Structural basis for pregnenolone biosynthesis by the mitochondrial monooxygenase system. *Proc Natl Acad Sci USA* **108**, 10139–10143.
- Beckert V & Bernhardt R (1997) Specific aspects of electron transfer from adrenodoxin to cytochromes P450_{sc} and P45011 β . *J Biol Chem* **272**, 4883–4888.
- Bowen AM, Johnson EO, Mercuri F, Hoskins NJ, Qiao R, McCullagh JS, Lovett JE, Bell SG, Zhou W, Timmel CR *et al.* (2018) A structural model of a P450-ferredoxin complex from orientation-selective double electron–electron resonance spectroscopy. *J Am Chem Soc* **140**, 2514–2527.
- Tripathi S, Li H & Poulos TLJS (2013) Structural basis for effector control and redox partner recognition in cytochrome P450. *Science* **340**, 1227–1230.

- 16 Grinberg AV & Bernhardt R (2001) Contribution of a salt bridge to the thermostability of adrenodoxin determined by site-directed mutagenesis. *Arch Biochem Biophys* **396**, 25–34.
- 17 Ringle M, Khatri Y, Zapp J, Hannemann F & Bernhardt R (2013) Application of a new versatile electron transfer system for cytochrome P450-based *Escherichia coli* whole-cell bioconversions. *Appl Microbiol Biotechnol* **97**, 7741–7754.
- 18 Khatri Y, Schiffrin A & Bernhardt R (2017) Investigating the effect of available redox protein ratios for the conversion of a steroid by a myxobacterial CYP260A1. *FEBS Lett* **591**, 1126–1140.
- 19 Zhang W, Liu Y, Yan J, Cao S, Bai F, Yang Y, Huang S, Yao L, Anzai Y & Kato F (2014) New reactions and products resulting from alternative interactions between the P450 enzyme and redox partners. *J Am Chem Soc* **136**, 3640–3646.
- 20 Hannemann F, Rottmann M, Schiffler B, Zapp J & Bernhardt R (2001) The loop region covering the iron-sulfur cluster in bovine adrenodoxin comprises a new interaction site for redox partners. *J Biol Chem* **276**, 1369–1375.
- 21 Uhlmann H & Bernhardt R (1995) The role of threonine 54 in adrenodoxin for the properties of its iron-sulfur cluster and its electron transfer function. *J Biol Chem* **270**, 29959–29966.
- 22 Uhlmann H, Iametti S, Vecchio G, Bonomi F & Bernhardt R (1997) Pro108 is important for folding and stabilization of adrenal ferredoxin, but does not influence the functional properties of the protein. *Eur J Biochem* **248**, 897–902.
- 23 Muller A, Muller JJ, Muller YA, Uhlmann H, Bernhardt R & Heinemann U (1998) New aspects of electron transfer revealed by the crystal structure of a truncated bovine adrenodoxin, Adx(4-108). *Structure* **6**, 269–280.
- 24 Kozakov D, Beglov D, Bohnuud T, Mottarella SE, Xia B, Hall DR & Vajda S (2013) How good is automated protein docking?. *Prot Struct Funct Bioinform* **81**, 2159–2166.
- 25 Kozakov D, Brenke R, Comeau SR & Vajda S (2006) PIPER: an FFT-based protein docking program with pairwise potentials. *Prot Struct Funct Bioinform* **65**, 392–406.
- 26 Sambrook J, Fritsch EF & Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- 27 Sligar SG & Gunsalus I (1976) A thermodynamic model of regulation: modulation of redox equilibria in camphor monooxygenase. *Proc Natl Acad Sci USA* **73**, 1078–1082.
- 28 Simgen B, Contzen J, Schwarzer R, Bernhardt R & Jung C (2000) Substrate binding to 15 β -hydroxylase (CYP106A2) probed by FT infrared spectroscopic studies of the iron ligand CO stretch vibration. *Biochem Biophys Res Comm* **269**, 737–742.
- 29 Schmitz D, Zapp J & Bernhardt R (2012) Hydroxylation of the triterpenoid dipterocarpol with CYP106A2 from *Bacillus megaterium*. *FEBS J* **279**, 1663–1674.
- 30 Lisurek M, Kang M-J, Hartmann RW & Bernhardt R (2004) Identification of monohydroxy progesterones produced by CYP106A2 using comparative HPLC and electrospray ionisation collision-induced dissociation mass spectrometry. *Biochem Biophys Res Comm* **319**, 677–682.
- 31 Schiffler B, Bureik M, Reinle W, Müller E-C, Hannemann F & Bernhardt R (2004) The adrenodoxin-like ferredoxin of *Schizosaccharomyces pombe* mitochondria. *J Inorg Biochem* **98**, 1229–1237.
- 32 Bureik M, Schiffler B, Hiraoka Y, Vogel F & Bernhardt R (2002) Functional expression of human mitochondrial CYP11B2 in fission yeast and identification of a new internal electron transfer protein, etp1 γ . *Biochemistry* **41**, 2311–2321.
- 33 Uhlmann H, Beckert V, Schwarz D & Bernhardt R (1992) Expression of bovine adrenodoxin in *E. coli* and site-directed mutagenesis of 2 FE-2S/cluster ligands. *Biochem Biophys Res Comm* **188**, 1131–1138.
- 34 Sagara Y, Wada A, Takata Y, Waterman MR, Sekimizu K & Horiuchi T (1993) Direct expression of adrenodoxin reductase in *Escherichia coli* and the Functional Characterization. *Biol Pharm Bull* **16**, 627–630.
- 35 Guex N & Peitsch MC (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* **18**, 2714–2723.
- 36 Comeau SR, Gatchell DW, Vajda S & Camacho CJ (2004) ClusPro: a fully automated algorithm for protein–protein docking. *Nucleic Acids Res* **32** (suppl 2), W96–W99.
- 37 Comeau SR, Gatchell DW, Vajda S & Camacho CJ (2004) ClusPro: an automated docking and discrimination method for the prediction of protein complexes. *Bioinformatics* **20**, 45–50.
- 38 Humphrey W, Dalke A & Schulten K (1996) VMD: visual molecular dynamics. *J Mol Graph* **14**, 33–38.
- 39 Jorgensen WL & Madura JD (1983) Quantum and statistical mechanical studies of liquids. 25. Solvation and conformation of methanol in water. *J Am Chem Soc* **105**, 1407–1413.
- 40 Berendsen HJ, Postma JV, vanGunsteren WF, DiNola A & Haak J (1984) Molecular dynamics with coupling to an external bath. *J Chem Phys* **81**, 3684–3690.
- 41 Parrinello M & Rahman A (1981) Polymorphic transitions in single crystals: a new molecular dynamics method. *J Appl Phys* **52**, 7182–7190.
- 42 Hess B (2008) P-LINCS: A parallel linear constraint solver for molecular simulation. *J Chem Theory Comput* **4**, 116–122.

2.3 Bakkes et al. (2017)

Engineering of versatile redox partner fusions that support monooxygenase activity of functionally diverse cytochrom P450s

Patrick J. Bakkes, Jan L. Riehm, Tanja Sagadin, Ansgar Rühlmann, Peter Schubert, Stefan Biemann, Marco Girhard, Michael C. Hutter, Rita Bernhardt, Vlada B. Urlacher

Scientific Reports, 7:9570, DOI:10.1038/s41598-017-10075-w (2017)

The author contributed to conversion experiments with different P450s.

SCIENTIFIC REPORTS

OPEN

Engineering of versatile redox partner fusions that support monooxygenase activity of functionally diverse cytochrome P450s

Received: 14 October 2016

Accepted: 4 August 2017

Published online: 29 August 2017

Patrick J. Bakkes¹, Jan L. Riehm², Tanja Sagadin³, Ansgar Rühlmann¹, Peter Schubert¹, Stefan Biemann¹, Marco Girhard¹, Michael C. Hutter², Rita Bernhardt³ & Vlada B. Urlacher¹

Most bacterial cytochrome P450 monooxygenases (P450s or CYPs) require two redox partner proteins for activity. To reduce complexity of the redox chain, the *Bacillus subtilis* flavodoxin YkuN (Y) was fused to the *Escherichia coli* flavodoxin reductase Fpr (R), and activity was tuned by placing flexible (GGGGS)_n or rigid ([E/L]PPPP)_n linkers (n = 1–5) in between. P-linker constructs typically outperformed their G-linker counterparts, with superior performance of YR-P5, which carries linker ([E/L]PPPP)₅. Molecular dynamics simulations demonstrated that ([E/L]PPPP)_n linkers are intrinsically rigid, whereas (GGGGS)_n linkers are highly flexible and biochemical experiments suggest a higher degree of separation between the fusion partners in case of long rigid P-linkers. The catalytic properties of the individual redox partners were best preserved in the YR-P5 construct. In comparison to the separate redox partners, YR-P5 exhibited attenuated rates of NADPH oxidation and heme iron (III) reduction, while coupling efficiency was improved (28% vs. 49% coupling with *B. subtilis* CYP109B1, and 44% vs. 50% with *Thermobifida fusca* CYP154E1). In addition, YR-P5 supported monooxygenase activity of the CYP106A2 from *Bacillus megaterium* and bovine CYP21A2. The versatile YR-P5 may serve as a non-physiological electron transfer system for exploitation of the catalytic potential of other P450s.

Cytochrome P450 monooxygenases (CYPs or P450s) are highly versatile heme containing enzymes that catalyse a wide variety of oxidation reactions, while accepting a large diversity of substrates. The impressive range of reactions catalysed by P450s includes amongst others hydroxylation, epoxidation, dealkylation and deamination, as well as unusual reactions such as aromatic dehalogenation and Baeyer-Villiger oxidation^{1–3}. Illustrative of their diverse biological functions, P450s are capable of converting amongst others fatty acids, steroids, prostaglandins, terpenes, and xenobiotics such as drugs and antibiotics^{4,5}. It is therefore not surprising that these multipurpose biocatalysts have become attractive targets for application in biotechnology and synthetic biology⁶.

P450s catalyse the reductive scission of molecular oxygen upon which one oxygen atom is introduced into the non-activated substrate, while the second atom is reduced to water⁷. Activation of molecular oxygen relies on the successive delivery of two electrons derived from the pyridine cofactor NAD(P)H to the heme iron, which is typically facilitated by a dedicated redox partner system^{8,9}.

Based on the number of proteins involved, two major types of P450 redox systems can be distinguished: (i) two-component systems consisting of an FAD-containing reductase and either an iron-sulfur containing ferredoxin or an FMN-containing flavodoxin. These systems are commonly found in bacteria and mitochondria, and (ii) a single diflavin (FAD/FMN) P450 reductase (CPR) that supports the function of eukaryotic microsomal P450s. A limited number of bacterial P450s have been identified that carry such a CPR-like module as a fused functional domain (for example CYP102A1 from *Bacillus subtilis*)^{8,9}.

¹Institute of Biochemistry, Heinrich-Heine University Düsseldorf, Universitätsstr. 1, 40225, Düsseldorf, Germany.

²Center for Bioinformatics, Saarland University, Campus Building E2.1, 66123, Saarbrücken, Germany. ³Institute of Biochemistry, Saarland University, Campus Building B2.2, 66123, Saarbrücken, Germany. Correspondence and requests for materials should be addressed to V.B.U. (email: vlada.urlacher@uni-duesseldorf.de)

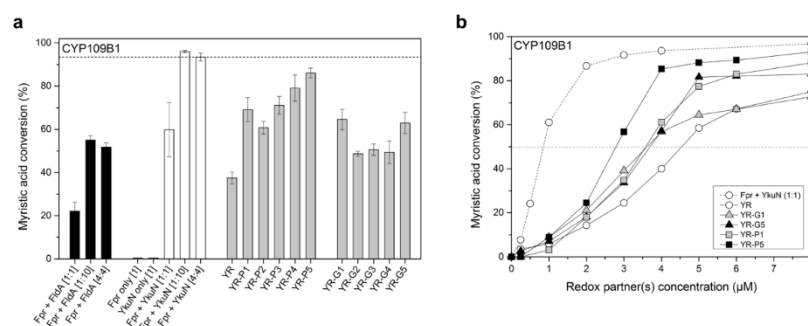


Figure 1. Myristic acid conversion by *B. subtilis* CYP109B1 supported by different redox partners. Reactions were started by the addition of a mixture of NADPH and myristic acid and allowed to proceed for 120 min under the support of an NADPH regenerating system. **(a)** CYP109B1 (1 μ M) conversion reactions were carried out in the presence of non-fused redox partners Fpr and FldA (black bars), Fpr and YkuN (white bars) or with different YkuN-Fpr fusion constructs (grey bars). Reductase (Fpr) and flavodoxin (FldA or YkuN) together with CYP109B1 were employed at respective ratios of 1:1:1, 1:10:1 and 4:4:1. Reactions conducted with YkuN-Fpr (YR) fusion constructs and CYP109B1 were carried out at a respective ratio of 4:1. YR indicates the linker-less YkuN-Fpr fusion construct, whereas linker designations P1 - P5 and G1 - G5 correspond to linker sequences (GGGGS)_n and (E/L)PPPP)_n of different lengths (n = 1–5). The data presented are average values of 3–6 independent conversion reactions with indicated standard deviation. **(b)** Myristic acid conversion by CYP109B1 in the presence of different concentrations of selected fusion constructs or non-fused Fpr/YkuN. The ratio of non-fused redox partners was maintained at 1:1.

The intrinsic dependence on NAD(P)H and redox partner(s) however limits the application and development of P450s as biocatalysts¹⁰. Moreover, identification of the physiological redox partner(s) of a given P450 is often hampered by the fact that host genomes usually contain numerous candidate genes coding for electron transfer proteins, which mostly are not located near the P450 gene(s)^{8,10}. Therefore surrogate electron transfer systems are frequently used for functional characterization and/or biocatalytic application of P450s. Cross-reactivity of eukaryotic P450s has been successfully exploited to support catalysis of a variety of mammalian enzymes¹¹, whereas two-component systems are frequently used to support the function of P450s from microorganisms. Notorious examples of these latter systems include putidaredoxin reductase (PdR) and putidaredoxin (Pdx) from *Pseudomonas putida*^{12,13}, bovine adrenodoxin reductase (AdR) and adrenodoxin (Adx)^{14,15} and flavodoxin/ferredoxin reductase (Fpr) from *E. coli* combined with either the flavodoxin FldA from *E. coli* or the flavodoxin YkuN from *Bacillus subtilis*^{16,17}. Moreover, in several cases electron transfer proteins could be covalently joined or even functionally attached to a P450^{18–25}. The principal aims of creating such artificial fusion constructs are to reduce complexity of the P450 systems and to improve the electron transfer properties to enhance catalytic performance^{11,26}.

Here, we report the construction of a set of redox fusion enzymes in which the flavodoxin YkuN from *B. subtilis* is fused to the N-terminus of the flavodoxin reductase Fpr from *E. coli*. To tune activity of the fused redox partners the linker region between them was varied in a systematic manner. Herein, both flexible (GGGGS)_n and rigid ([E/L]PPPP)_n linkers of different lengths (n = 1–5) were introduced between YkuN and Fpr by using the previously established DualInX tool¹⁹. After expression in *E. coli* and subsequent purification, the activity of the fusion constructs was investigated with respect to their ability to oxidize NADPH and reduce the P450 heme iron, as well as their capability to support the monooxygenase activity of a variety of P450s *in vitro*.

Results

Construction of redox partner fusion enzymes consisting of *B. subtilis* YkuN and *E. coli* Fpr

The flavodoxin YkuN from *Bacillus subtilis* is a promiscuous electron carrier capable of transferring electrons to a variety of P450s, including endogenous CYP107H1 (P450 BioI) and CYP109B1, but also to heterologous P450s such as CYP154A8 from *Nocardia farcinica* or CYP154E1 from *Thermobifida fusca* YX^{16,17,27}. Herein, YkuN is typically paired with the flavodoxin reductase Fpr from *E. coli*, which provides the NADPH-derived electrons. The “mixed” redox pair Fpr/YkuN often outperforms physiological redox pairs such as *E. coli* Fpr/FldA or *P. putida* PdR/Pdx^{16,17}. Consistent with this, Fpr/YkuN supported substantially higher conversion of myristic acid by CYP109B1 than the Fpr/FldA system (Fig. 1a).

Importantly, even in the case of natural P450 redox chains, such as PdR:Pdx:P450cam, excess of the ferredoxin Pdx is required for productive catalysis *in vitro*²⁸. Similarly, for Fpr/YkuN/P450 systems, a respective stoichiometry of 1:10:1 is usually sufficient to efficiently drive P450-mediated catalysis *in vitro*^{16,17}. Thus surplus of flavodoxin or ferredoxin overcomes apparent rate-limiting steps in electron transfer^{16,28,29}. In support of this, a 1:10:1 Fpr/YkuN/CYP109B1 system achieved 36% higher conversion of myristic acid than a 1:1:1 system (Fig. 1a), which is consistent with the function of YkuN as an electron shuttle that transfers the NADPH-derived electrons

from the reductase to the P450^{27,29}. A stimulatory effect was also noted for the alternative system using the *E. coli* flavodoxin FldA (Fig. 1a). Nevertheless, independent of the stoichiometry applied the Fpr/YkuN redox pair proved to be superior to Fpr/FldA in supporting CYP109B1 catalysis, which makes YkuN a promising candidate for covalent fusion to Fpr.

YkuN was fused to the N-terminus of Fpr, leaving the C-terminus of Fpr free, as it was demonstrated previously that attachment of FldA to the C-terminus of Fpr leads to reduced enzyme function, whereas constructs with the fusion partners in reversed order were substantially more active¹⁹. Attachment of the flavodoxin to the C-terminus of Fpr likely interferes with the function of the aromatic residues at the extreme C-terminus of Fpr, which are important for NADPH binding and electron exchange^{30–32}. It is worthy of note that homologous catalytic residues within the reductase domain of natural fusion proteins such as CPR and self-sufficient P450s such as CYP102A1 are also found at the C-terminus^{6,9}.

To optimize activity of the YkuN-Fpr (YR) fusion construct both flexible (GGGGG)_n and rigid ([E/L]PPPP)_n linkers of different lengths (n = 1–5) were placed between the fusion partners (for linker specifications see Supplementary Table S2). Thus, 11 different fusion constructs were created that were expressed in *E. coli*. The corresponding fusion proteins were subsequently isolated from the soluble protein fraction and purified to near homogeneity by IMAC (Supplementary Fig. S1), typically yielding 1–2 mg of purified fusion protein from 40 ml culture (OD₆₀₀ = 4–5).

YkuN-Fpr fusion constructs support monooxygenase activity of CYP109B1. The ability of the fusion constructs (4 μM) to support P450 monooxygenase activity was tested *in vitro* with CYP109B1 (1 μM) using myristic acid as substrate (Fig. 1a). Because the stoichiometry of the fused redox partners is fixed at 1:1, control reactions were conducted with 4 μM each of non-fused Fpr and YkuN. Independent of the flavodoxin used (FldA or YkuN), myristic acid conversion achieved with the 4:4:1 reconstituted system was nearly equal to that obtained with the commonly used 1:10:1 system (Fig. 1a). Importantly, the different fusion constructs were all able to support CYP109B1 catalysis (Fig. 1a), which indicates that the covalently attached Fpr and YkuN have retained their ability to deliver the necessary electrons to CYP109B1. The linker-less fusion YR exhibited appreciable activity, supporting ~38% conversion of myristic acid (Fig. 1). Nevertheless, conversion was substantially lower than with equivalent amounts of non-fused Fpr/YkuN, suggesting reduced functionality upon direct attachment of the redox partners. Introduction of a linker between YkuN and Fpr however substantially improved activity. In particular the fusion constructs YR-P4 and YR-P5 supported high myristic acid conversion (79% and 86%, respectively), which is close to the 94% conversion achieved with non-fused Fpr/YkuN (Fig. 1a). Quantitative product analysis revealed that both the regioselectivity of CYP109B1 for myristic acid hydroxylation as well as product distribution was not affected by the fusion constructs (Supplementary Table S3). In all cases, the carbon atoms C-11 (ω₋₃) and C-12 (ω₋₂) of myristic acid were preferentially hydroxylated.

Interestingly, the fusion constructs carrying proline-rich linkers (P1–P5) in all cases outperformed their glycine-rich (G1–G5) counterparts (Fig. 1a). Moreover, the performance of the P-linker constructs seems to be dependent on the linker length, supporting higher conversion with increased linker length, whereas for the G-linker constructs a dependence on linker length was not evident (Fig. 1a).

Spectral analysis of the different YkuN-Fpr fusion constructs revealed nearly identical absorbance characteristics (Supplementary Fig. S3), which indicates that the fusion proteins have a similar cofactor content (Fpr-FAD and YkuN-FMN) as well as a similar cofactor-protein environment. It seems therefore unlikely that the differences in activity between the fusion constructs relate to linker induced effects on cofactor binding or protein folding.

The performance of the fusion constructs was further investigated using different redox protein concentrations (0–8 μM). In all cases, sigmoid curves were obtained over the range of concentrations tested (Fig. 1b). The dependence of CYP109B1 activity on the fusion protein concentration was clearly different from that of an equivalent system of non-fused Fpr/YkuN maintained at a 1:1 ratio, and also differed among the fusion constructs, which indicates rather complex behaviour (Fig. 1b).

Overall, YR-P5 proved the most effective fusion construct, as it supported nearly complete conversion of myristic acid at protein concentrations of 4–8 μM, much like the non-fused redox partners. However, at concentrations <4 μM, the performance of YR-P5 dropped well below that of the non-fused Fpr/YkuN, as was also observed for the other tested fusion constructs (Fig. 1b). As a measure for the overall performance of the redox partners, the concentration of the redox enzymes at which 50% of myristic acid (EC₅₀) was converted by CYP109B1 was estimated from Fig. 1b. The approximate EC₅₀ are 0.8 μM for non-fused Fpr/YkuN, 2.7 μM for YR-P5, 3.5 μM for both YR-P1 and YR-G1, 3.6 μM for YR-G5 and 4.5 μM for the linker-less construct YR, which demonstrates that YR-P5 is the superior fusion construct.

Intrinsic properties of the (GGGGG)_n and ([E/L]PPPP)_n linkers. To further evaluate the properties of the (GGGGG)_n and ([E/L]PPPP)_n linkers molecular dynamics (MD) simulations were carried out. MD simulations of the G-linkers revealed that starting from a linear extended conformation these linkers readily fold into more compact random structures (Fig. 2a). Collapse of the extended conformation is particularly evident for linkers G3, G4 and G5, which consist of 3, 4 and 5 (GGGGG)-repeats, respectively. These linkers exhibited a substantial (>1 Å) decrease in the radius of gyration (Rg) over time (Fig. 2b). Moreover, the Rg of the G4 and G5 linker exhibited large oscillations over time, which indicates substantial structural rearrangements and high structural flexibility. Structural behaviour of the ([E/L]PPPP)_n linkers on the other hand was clearly different. The Rg of the different P-linkers remained largely constant over time, with only minor oscillations observed for linkers P3–P5 (Fig. 2c). Thus, the P-linkers effectively maintained their linear conformation. Consequently, the Rg of the P-linkers exhibited a dependence on the linker length. For each additional ([E/L]PPPP)-segment the Rg increased by ~0.4–0.5 Å (Fig. 2c). Linker P5, which consists of 25 residues forms an exception to this as it

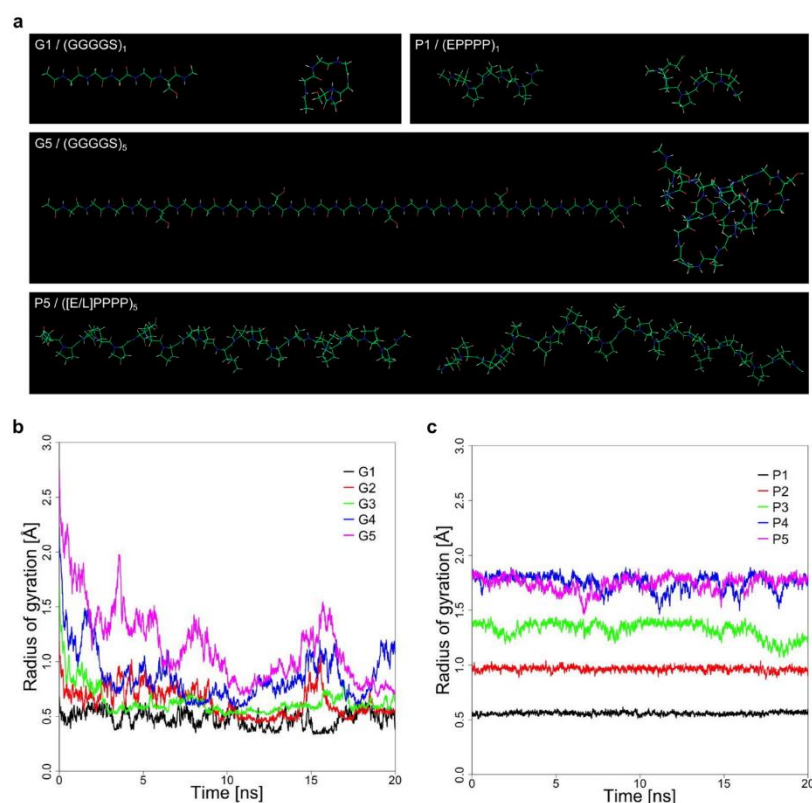


Figure 2. MD simulations of the (GGGGS)_n and ([E/L]PPPP)_n linkers used to functionally connect YkuN and Fpr. (a) Structural snapshots of (GGGGS)_n and ([E/L]PPPP)_n linkers of length 1 and 5. Depicted in each panel are the starting conformations (*left*) and the lowest energy conformation found during the 20 ns MD simulations (*right*). Development of the radius of gyration (Rg) during MD simulations for (GGGGS)_n and ([E/L]PPPP)_n linkers of lengths (n = 1–5) are shown in (b) and (c), respectively. The strong decrease in Rg for (GGGGS)_n linkers reflects the hydrophobic collapse, whereas the ([E/L]PPPP)_n linkers remain in an extended conformation.

exhibited an Rg similar to that of the P4 linker, i.e. ~1.8 Å (Fig. 2c). Overall bending of the long P-linkers may have contributed to this occurrence (Fig. 2a).

Influence of auxiliary YkuN on CYP109B1 catalysis driven by the YkuN-Fpr fusion constructs.

In comparison to the separate redox enzymes the fusion constructs exhibit a reduced performance at low protein concentration (Fig. 1b), which may indicate that one (or both) of the fused redox partners is limiting the P450-catalysed reaction. With reconstituted P450 systems the electron shuttle ferredoxin/flavodoxin is usually a major limiting factor that is typically overcome by applying an excess of this electron transfer protein^{16, 28, 29}. To investigate this, the different fusion constructs (1 μM) were incubated together with CYP109B1 (1 μM) in the absence or presence of an excess of YkuN (4 or 9 μM). In all cases, mixing the fusion proteins with additional YkuN led to an increase in myristic acid conversion (Fig. 3), which confirms that YkuN is limiting the reaction. By increasing the concentration of the fusion constructs itself in the P450-catalysed reaction, this apparent limitation was also (partly) overcome (Fig. 1b).

Since the reductase (Fpr) domain of the fusion constructs is capable of donating electrons to the auxiliary electron shuttle YkuN, this also indicates that no strict functional coupling between the Fpr and YkuN domain exists within the fusion constructs. Generally, electron transfer rates are thought to be controlled in part by the distance between the redox centres³³. Considering the YkuN-Fpr fusion constructs it is likely that both the length and the structural properties of the linker contribute to the relative distance between the respective redox centres. Regarding the rigid nature of the ([E/L]PPPP)_n linkers (Fig. 2) it seems plausible that in particular long P-linkers

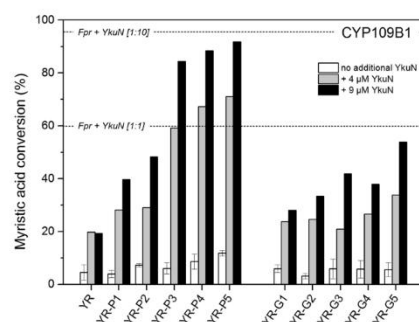


Figure 3. Influence of auxiliary YkuN on CYP109B1 catalysis driven by the different YkuN-Fpr fusion constructs. Myristic acid conversion with CYP109B1 (1 μ M) was carried out in the presence of the different YkuN-Fpr fusion constructs either in the absence (- YkuN) or presence of additional YkuN (4 or 9 μ M). In all cases reactions were started by the addition of a mixture of NADPH and myristic acid and allowed to proceed for 120 min under the support of an NADPH regenerating system. The Fpr/YkuN/CYP109B1 system reconstituted at 1:10:1 and 1:1:1 ratio, respectively achieved 96% and 60% conversion of myristic acid in 120 min.

Reconstituted system ^a	NADPH oxidation rate ^b	Coupling ^c efficiency (%)
Fpr / YkuN / CYP109B1 - [4:4:1]	26.0 $\pm$ 1.9	28.3 $\pm$ 3.6
YR / CYP109B1 - [4:1]	3.1 $\pm$ 0.3	49.8 $\pm$ 8.3
YR-P1 / CYP109B1 - [4:1]	12.7 $\pm$ 1.3	62.1 $\pm$ 5.1
YR-P5 / CYP109B1 - [4:1]	11.3 $\pm$ 1.9	48.9 $\pm$ 3.4
YR-G1 / CYP109B1 - [4:1]	6.6 $\pm$ 0.3	72.4 $\pm$ 3.6
YR-G5 / CYP109B1 - [4:1]	1.4 $\pm$ 0.2	81.2 $\pm$ 4.1

Table 1. NADPH oxidation rate and coupling efficiency of the CYP109B1-catalysed conversion of myristic acid, supported by different redox partners. Presented data represent average values of at least three independent reactions. The ratio of NADPH:myristic acid employed was 1:1 (200 μ M each, respectively). ^aValues in brackets indicate applied ratio as well as final concentration (μ M) of indicated proteins. ^bRates are given in nmol NADPH per nmol CYP109B1 per minute. The background NADPH oxidation rate in the absence of redox partner(s) was 0.1 $\pm$ 0.0. ^cThe coupling efficiency equals the myristic acid conversion achieved upon NADPH depletion (see also Supplementary Fig S5).

might facilitate a higher degree of separation of the fusion partners. In line with this, the increase in conversion upon YkuN addition was much more pronounced for fusion constructs carrying the rigid P-linkers (Fig. 3), which more effectively maintain an extended conformation than their glycine-rich counterparts (Fig. 2). In fact, the highest increase in myristic acid conversion governed by the addition of YkuN was observed for the YR-P5 construct which carries the longest P-linker tested (25 residues), achieving a similar conversion as the comparable 1:10 system of non-fused Fpr/YkuN (Fig. 3).

NADPH oxidation rates and coupling efficiencies measured with the YkuN-Fpr fusion enzymes. The overall performance of a reconstituted P450-redox partner system depends amongst others on its NAD(P)H oxidation rate and the coupling efficiency between NAD(P)H oxidation and P450 substrate conversion. Since delivery of electrons to CYP109B1 depends on functional reductase (Fpr) as well as flavodoxin (YkuN), a loss of electrons may occur either upon electron transfer from Fpr to YkuN or from YkuN to CYP109B1, which in both cases will lead to uncoupling between NADPH oxidation and product formation.

The NADPH oxidation rate and the coupling efficiency were determined for various reconstituted systems (Table 1). CYP109B1 reconstituted with the non-fused redox partners exhibited the highest NADPH oxidation rate, i.e. 26 nmol·(nmol P450)⁻¹·min⁻¹, along with a coupling efficiency of 28.3%. In contrast, for the fusion constructs substantially lower NADPH consumption rates were observed, whereas the coupling efficiency was markedly improved (Table 1). Direct attachment of YkuN to Fpr (YR) led to a ~1.8-fold improvement of the coupling efficiency (49.8%) and a more than 8-fold decrease in NADPH oxidation rate (Table 1). In comparison to YR, the superior construct YR-P5 (Fig. 1) showed a similar coupling (48.9%), whereas the NADPH oxidation rate was 3.6-fold higher, i.e. 11.3 nmol·(nmol CYP)⁻¹·min⁻¹ (Table 1). The highest coupling efficiency of 81.2% was however observed for YR-G5, which, notably, exhibited the lowest NADPH oxidation rate of 1.4 nmol·(nmol P450)⁻¹·min⁻¹.

Reconstituted system ^a	Heme-iron reduction rate ^b	
	k_1 (s ⁻¹)	k_2 (s ⁻¹)
Fpr / FldA / CYP109B1 – [4:4:1]	0.004 ± 0.001	0.027 ± 0.008
Fpr / YkuN / CYP109B1 – [4:4:1]	0.009 ± 0.001	0.078 ± 0.023
YR / CYP109B1 – [4:1]	0.002 ± 0.001	0.030 ± 0.002
YR-P5 / CYP109B1 – [4:1]	0.008 ± 0.002	0.045 ± 0.005
YR-G5 / CYP109B1 – [4:1]	0.007 ± 0.001	0.035 ± 0.002

Table 2. Reduction of CYP109B1 Fe³⁺-heme by different redox partners. ^aValues in brackets indicate applied ratio as well as final concentration (μM) of the proteins in the employed reconstituted systems. ^bThe heme-iron reduction rates were measured as described in the Methods section. Kinetic traces were fit to a bi-exponential function, revealing a slow and a fast phase. Typical kinetic traces and corresponding fits are shown in the online Supplementary Information. Presented reduction rates represent average values of at least three independent reactions carried out under anaerobic conditions at 20 °C.

Reduction of CYP109B1 Fe³⁺-heme by the YkuN-Fpr fusion constructs. Since the YkuN-Fpr fusion constructs exhibited attenuated NADPH oxidation rates along with improved coupling, the question arises whether the crucial step of electron transfer to the P450 is also influenced upon attachment of YkuN to Fpr. The reduction of the Fe³⁺ to Fe²⁺-heme can be conveniently monitored by the addition of carbon monoxide (CO), yielding a stable complex that exhibits the characteristic absorbance maximum at 450 nm³⁴.

Indeed, absorbance spectroscopy of the anaerobic reduction of CYP109B1 in the presence of CO by the different redox partners revealed the formation of the typical absorbance peak at ~450 nm, which is governed by the reduction of Fe³⁺ to Fe²⁺-heme and the subsequent formation of the stable heme-Fe²⁺-CO complex. Typical traces for the time dependent reduction are shown in Supplementary Fig. S4. P450 reduction occurred rather slowly under the tested conditions (Fig. S4). Data were fit to a bi-exponential function, revealing a slow and a fast phase; the corresponding reduction rates are shown in Table 2. Reduction occurred fastest by the non-fused Fpr/YkuN, exhibiting a k_1 of 0.009 s⁻¹ and k_2 of 0.078 s⁻¹. For the linker-less fusion YR corresponding rates were 4.5 and 2.6-fold decreased. In contrast, for the fusion constructs carrying the long P5 or G5 linker, k_1 was virtually identical to that of the non-fused Fpr/YkuN and k_2 was decreased to a lesser extent (Table 2). Of the tested fusion constructs, the superior YR-P5 also exhibited the highest heme-iron reduction rate (k_1 0.008 s⁻¹ and k_2 0.045 s⁻¹). Thus, introduction of the P5 linker (25 residues) between the fused YkuN and Fpr led to a substantial improvement of the heme-iron reduction rate.

Versatility of the YkuN-Fpr redox fusion enzymes. From a biotechnological point of view, a redox partner system desirably should have the ability to transfer electrons to different terminal acceptors. To demonstrate cross-reactivity, selected fusion constructs were additionally tested with CYP154E1 from *Thermobifida fusca* YX, CYP106A2 from *Bacillus megaterium* ATCC 13368 and bovine CYP21A2.

CYP154E1 is a versatile monooxygenase that converts a large variety of substrates, including fatty acids and alcohols, as well as acyclic and bulky cyclic terpenoids^{17,35,36}. Since Fpr/YkuN are known to effectively support CYP154E1 catalysis^{17,35,36} it is expected that the YkuN-Fpr fusion constructs are also able to act as surrogate redox partners. To investigate this, selected fusion constructs were tested for their ability to support CYP154E1 catalysis using β-ionone as substrate (Fig. 4). Ionones are cyclic terpenoids that are key fragrance components used for the production of perfumes, cosmetics and other fine chemicals³⁷. Moreover, β-ionone is an important intermediate in the manufacturing of vitamin A, while its oxygenated derivative 4-hydroxy-β-ionone is a key intermediate in the synthesis of carotenoids and the plant hormone abscisic acid^{38–40}.

Non-fused Fpr/YkuN (1:10 or 4:4 system) supported CYP154E1 catalysis, achieving >70% conversion of β-ionone in 1 h (Fig. 4). Thus, in addition to the previously reported terpenoid substrates³⁶, β-ionone is also converted by CYP154E1. As expected, the fusion constructs were able to support CYP154E1 catalysis, of which YR-P5 again proved most effective at driving P450 catalysis (Fig. 4). In the presence of YR-P5 ~1 mM of β-ionone was converted in 1 h, yielding a turnover of ~16 min⁻¹, while non-fused Fpr/YkuN (4:4) supported a turnover of ~24 min⁻¹. The YR-P5/CYP154E1 (4:1) system achieved a coupling efficiency of ~50%, whereas with the non-fused enzymes ~44% was measured (Supplementary Table S4). Furthermore, under cofactor regeneration conditions, total turnover numbers (TTN) were 1,582 with YR-P5 and 1,948 with the non-fused Fpr/YkuN (Supplementary Table S5). Regardless whether Fpr and YkuN were fused or not, in all cases a major oxidation product was formed, which was identified as 4-hydroxy-β-ionone by comparison to MS analysis of an authentic reference substance⁴¹.

To further substantiate functional promiscuity, the best construct YR-P5 was tested with CYP106A2 and CYP21A2 using progesterone as substrate in both cases. It is of note that these P450s have not been tested previously with Fpr/YkuN as surrogate redox partners. CYP106A2 is a regio- and stereoselective 15β-hydroxylase of 3-oxo-Δ⁴-steroids^{42,43} that recently has been demonstrated to also accept 3-hydroxy-Δ⁵-steroids as well as di- and triterpenes as substrates^{44,45}. Herein, the bovine adrenodoxin reductase (AdR) and adrenodoxin (Adx) typically serve as surrogate redox partners. Interestingly Fpr/YkuN and its fused derivative YR-P5 were able to functionally substitute for AdR/Adx in the CYP106A2-mediated conversion of progesterone (Table 3). Moreover, performance of the 4:4:1 Fpr/YkuN/CYP106A2 system was virtually identical to that of the 4:4:1 AdR/Adx/CYP106A2 system, both with respect to progesterone conversion and product distribution (Table 3). With both systems nearly all progesterone was converted after 120 min and 15β-hydroxyprogesterone was the main product

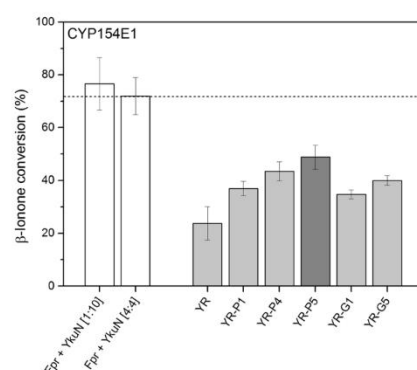


Figure 4. Conversion of β -ionone by *T. fusca* YX CYP154E1 supported by selected fusion constructs. Reactions were carried out in the presence of 1 μ M CYP154E1 and 4 μ M of indicated fusion constructs. Control reactions with non-fused Fpr/YkuN were conducted with 1 μ M CYP154E1 together with either 1 μ M Fpr + 10 μ M YkuN [1:10], or 4 μ M Fpr + 4 μ M YkuN [4:4]. In all cases the initial concentration of the substrate β -ionone was 2 mM. Reactions were started by the addition of NADP⁺ under support of an NADPH regenerating system and stopped after 1 h.

Redox partner(s)	Ratio ^a	Conversion time (min)	Progesterone conversion (%)	Compounds (%)			
				Prog. ^b	15 β -OH ^b	mono-OH ^b	poly-OH ^b
AdR/Adx	(4:4:1)	30	96.0 $\pm$ 0.5	4.0 $\pm$ 0.5	81.7 $\pm$ 1.0	8.7 $\pm$ 1.3	5.6 $\pm$ 0.9
		120	99.0 $\pm$ 0.1	1.0 $\pm$ 0.1	81.1 $\pm$ 0.5	6.9 $\pm$ 0.5	11.0 $\pm$ 0.7
	(10:10:1)	30	96.0 $\pm$ 0.6	4.0 $\pm$ 0.6	81.5 $\pm$ 0.5	7.9 $\pm$ 0.1	6.6 $\pm$ 0.2
		120	96.7 $\pm$ 1.0	3.3 $\pm$ 1.0	82.6 $\pm$ 1.6	7.9 $\pm$ 0.3	6.1 $\pm$ 1.4
Fpr/YkuN	(4:4:1)	30	92.7 $\pm$ 0.7	7.3 $\pm$ 0.7	80.4 $\pm$ 0.5	4.0 $\pm$ 0.4	8.3 $\pm$ 0.1
		120	98.9 $\pm$ 0.0	1.1 $\pm$ 0.0	81.9 $\pm$ 0.0	4.3 $\pm$ 0.0	12.8 $\pm$ 0.0
	(10:10:1)	5 (500 μ M) ^c	84.7 $\pm$ 5.4	15.3 $\pm$ 5.4	80.4 $\pm$ 3.1	2.0 $\pm$ 0.2	2.2 $\pm$ 2.2
		30	91.5 $\pm$ 1.3	8.5 $\pm$ 1.3	63.0 $\pm$ 1.0	4.4 $\pm$ 0.1	24.1 $\pm$ 0.2
		60	94.0 $\pm$ 0.1	6.0 $\pm$ 0.1	58.4 $\pm$ 0.7	5.2 $\pm$ 0.7	30.5 $\pm$ 0.1
		90	98.5 $\pm$ 0.4	1.5 $\pm$ 0.4	68.2 $\pm$ 4.2	6.5 $\pm$ 0.4	23.8 $\pm$ 4.7
		120	97.8 $\pm$ 0.2	2.1 $\pm$ 0.2	66.3 $\pm$ 2.6	6.4 $\pm$ 0.4	25.2 $\pm$ 3.0
	(10:1)	30	19.5 $\pm$ 2.6	80.5 $\pm$ 2.6	19.1 $\pm$ 2.1	0.4 $\pm$ 0.5	—
		60	29.6 $\pm$ 5.4	70.4 $\pm$ 5.4	29.2 $\pm$ 4.9	0.4 $\pm$ 0.6	—
		90	39.7 $\pm$ 16.2	60.3 $\pm$ 16.2	39.7 $\pm$ 16.2	—	—
		120	45.9 $\pm$ 15.2	54.1 $\pm$ 15.2	44.0 $\pm$ 13.4	1.9 $\pm$ 1.9	—
		5 (500 μ M) ^c	29.7 $\pm$ 0.6	70.3 $\pm$ 0.6	29.7 $\pm$ 0.6	—	—
		30	75.9 $\pm$ 3.8	24.1 $\pm$ 3.8	71.3 $\pm$ 4.2	3.4 $\pm$ 0.5	1.2 $\pm$ 0.9
		60	80.0 $\pm$ 0.9	20.0 $\pm$ 0.9	74.9 $\pm$ 1.4	3.2 $\pm$ 0.8	1.8 $\pm$ 0.3
		90	90.7 $\pm$ 5.3	9.3 $\pm$ 5.3	81.5 $\pm$ 3.7	3.6 $\pm$ 0.8	5.6 $\pm$ 1.0
		120	94.9 $\pm$ 0.7	5.1 $\pm$ 0.7	84.9 $\pm$ 0.7	4.0 $\pm$ 1.3	6.1 $\pm$ 0.2

Table 3. Product distribution for the CYP106A2-catalysed conversion of progesterone supported by different redox partners. Data represent average values of three independent reactions (using 200 μ M progesterone) with indicated standard deviation. ^aRedox partner-CYP106A2 ratio of reconstituted system. ^b*B. megaterium* CYP106A2 hydroxylates progesterone at positions 15 β , 6 β , 11 α and 9 α ⁷⁶; mono-OH, monohydroxylated progesterone at positions other than 15 β ; poly-OH, di- or polyhydroxylated progesterone. ^cTo assess turnover, conversion reactions were carried out for 5 min using 500 μ M progesterone.

formed (Table 3). The fusion construct YR-P5 also supported CYP106A2 catalysis, albeit with lower efficacy (Table 3). The YR-P5/CYP106A2 (4:1) system achieved ~46% conversion after 120 min, producing almost exclusively 15 β -OH-progesterone (Table 3). By increasing the YR-P5 concentration (10:1 system) conversion could be enhanced to near completion (95%) after 120 min, while only minor amounts of undesired polyhydroxylated progesterone were formed (up to 6%).

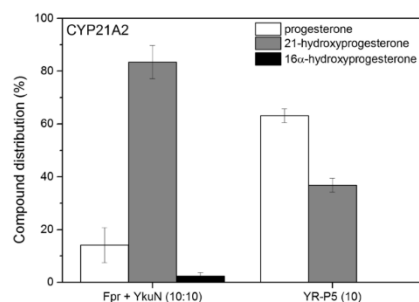


Figure 5. Conversion of progesterone by bovine CYP21A2 supported by Fpr/YkuN and their fused derivative YR-P5. In all cases reactions were carried out in the presence of 0.5 μ M CYP21A2, 200 μ M progesterone and 5 μ M of indicated redox partner(s). Corresponding ratios of redox partner(s) relative to CYP21A2 are indicated in brackets. Reactions were started by the addition of NADPH and supported by a NADPH regenerating system. Reactions were stopped after 30 min.

To assess turnover rates, reactions were carried out under conditions in which essentially no overoxidation products were formed (5 min reactions using 500 μ M progesterone). With the Fpr/YkuN/CYP106A2 system (10:10:1) nearly 85% of the progesterone was converted, thus achieving a turnover of 169 min^{-1} , whereas with the YR-P5/CYP106A2 system (10:1) nearly 30% progesterone conversion was achieved, thus yielding a turnover of 59.4 min^{-1} .

To further illustrate versatility of YR-P5, bovine CYP21A2, which is a membrane-bound microsomal P450, was used as terminal electron acceptor. CYP21A2 is involved in the biosynthesis of steroid hormones, and typically catalyses the hydroxylation of the carbon atom 21 in steroids⁴⁶. In general, CYP21A2 obtains the necessary electrons from NADPH via an FAD and FMN-containing microsomal CPR⁸. However, it is also able to use AdR and Adx as redox partners⁴⁷. Notably, YR-P5 supported CYP21A2 catalysis; with YR-P5 40% of the (200 μ M) progesterone was converted to 21-hydroxyprogesterone in 30 min, while ~80% conversion was achieved with non-fused Fpr/YkuN (Fig. 5). Taken together, the Fpr/YkuN redox pair and its fused derivative YR-P5 are able to support the activity of the steroidogenic CYP106A2 and CYP21A2 *in vitro*, which indicates that these bacterial electron transfer systems may serve as alternatives to the mammalian electron transfer systems AdR/Adx and CPR.

Discussion

Flavodoxins are promiscuous electron carriers that donate electrons to structurally and functionally diverse enzymes, including pyruvate-formate lyase⁴⁸, ribonucleotide reductase⁴⁹, key enzymes in photosynthesis⁵⁰, nitrogen fixation⁵¹, methionine⁵² and biotin⁵³ synthesis, but also P450s^{16,27,29,54,55}. The mixed redox pair Fpr/YkuN outperformed the physiological redox pair Fpr/FldA from *E. coli* in supporting CYP109B1 catalysis (Fig. 1a), which suggests that electron transfer by the flavodoxin YkuN is more effective. Since YkuN and CYP109B1 both originate from *B. subtilis* it is possible that YkuN is the physiological redox partner of CYP109B1 and therefore a more favourable electron carrier than the heterologous FldA.

Typically, the midpoint potentials of the oxidised/semiquinone (E_1') and semiquinone/hydroquinone (E_2') couples reported for the short-chain flavodoxin YkuN are higher than those for the long-chain flavodoxin FldA, i.e. -105 mV and -382 mV²⁷ vs. -254 mV and -433 mV, respectively⁵⁵. E_1' and E_2' of the employed *E. coli* reductase Fpr are -308 mV and -268 mV, respectively⁵⁵. Indeed, the anaerobic reduction of CYP109B1 heme iron by YkuN occurred 2–3 times faster than by FldA (Table 2). Similarly, stopped-flow experiments monitoring the reduction of *B. subtilis* CYP107H1 have indicated that YkuN is more effective than FldA in first electron transfer to palmitoleate-bound CYP107H1, as evidenced by a ~12-fold higher k_{red} ²⁷. Thus, accelerated electron transfer likely contributed to the overall better performance of YkuN compared to FldA in the CYP109B1-catalysed conversion of myristic acid (Table 2, Fig. 1a).

The inherent dependence of P450s on electron transfer proteins presents a challenging limitation in their biotechnological exploitation. To simplify redox chains and to improve the catalytic properties of P450 systems, a variety of man-made P450 fusion enzymes have been successfully created using a variety of molecular approaches, including “Molecular Lego”²², “LICRED”⁵⁶, and “PUPPET”⁵⁷. Here, the versatility and superior properties of YkuN were exploited to generate redox fusion enzymes capable of driving catalysis of different CYPs, using the previously established DualInX procedure¹⁹ for linker engineering. The catalytic performance of a reconstituted P450 system is often limited by a low coupling efficiency. Uncoupling events waste expensive reduced cofactors (NAD(P)H) and lead to the generation of reactive oxygen species that can cause enzyme inactivation⁵⁸. With non-physiological redox chains the coupling efficiency is often particularly poor (less than 20%)¹⁶ and also when P450s catalyse reactions with non-physiological substrates, coupling efficiencies are frequently severely diminished (<10%)⁵⁸. Achieving a high coupling efficiency is thus a particularly challenging task.

Direct attachment of YkuN to Fpr via genetic fusion led to a 1.8-fold improvement of the coupling efficiency in CYP109B1-catalysed reactions (Table 1). Coupling efficiency could be even further improved by insertion of

an appropriate linker between the fusion partners. In case of the YR-G5 construct a coupling efficiency as high as 81.2% was achieved. Notably, in all cases the CYP109B1 systems reconstituted with the different YkuN-Fpr fusion constructs exhibited a higher coupling efficiency than those reported previously using a variety of different redox partners (1.8–45.8%)¹⁶, which is an advantage under cofactor regeneration condition. However, the NADPH oxidation rate was markedly reduced.

Despite improved coupling, overall performance of the parental linker-less YkuN-Fpr fusion construct in P450-catalysed reactions was rather modest in comparison to the non-fused enzymes (Figs 1 and 4). A > 8-fold reduction in NADPH oxidation rate (Table 1) combined with slower electron transfer to the CYP109B1 (Table 2) likely contributed to the reduced performance of the YR fusion construct. Insertion of a suitable linker between the fusion partners however, substantially improved the overall performance. Herein, long ([E/L]PPPP)_n linkers (n = 4–5) were particularly effective (Figs 1 and 4), which is consistent with previous findings for fusions between *E. coli* FldA and Fpr¹⁹. At protein concentrations ≥ 4 μM, the superior construct YR-P5 acted as a nearly equivalent substitute for non-fused Fpr/YkuN in driving CYP109B1 catalysis (Fig. 1). Insertion of the P5 linker between the fused YkuN and Fpr resulted in a 1.5-fold increase in heme iron reduction rate (Table 2, *k*₂) along with a 3.6-fold higher NADPH oxidation rate, while maintaining a high coupling efficiency of nearly 50% (Table 1), thus contributing to the improved overall performance (Fig. 1). However, despite poorer coupling efficiency, the non-fused redox partners proved generally more effective than YR-P5 in supporting P450 catalysis (Table 3, Figs 1, 4 and 5), which is likely governed by faster overall electron transfer (accelerated NADPH oxidation as well as P450 reduction). The higher amount of progesterone overoxidation products formed by CYP106A2 when the non-fused redox partners are used as opposed to YR-P5, is also consistent with this notion (Table 3).

Examination of the linker properties using MD simulations revealed that the (GGGG)_n linkers are highly flexible and tend to adopt compact random structures, whereas the ([E/L]PPPP)_n linkers maintain their linear conformation and are therefore structurally more rigid (Fig. 3). These results are consistent with the notion that glycine-rich and proline-rich amino acid sequences are flexible and rigid in nature, respectively^{59–61}. Moreover, the high mobility of glycine-rich linkers is apparent from their lack of structural resolution in X-ray structures of a multitude of artificial fusion proteins⁶². Due to their flexibility, glycine-rich linkers are unstructured and tend to provide limited domain separation^{63,64}, whereas structurally rigid linkers, such as proline-rich linkers are more likely to separate the fusion partners^{60,65}.

For flavodoxins the binding areas for the electron donating reductase and the terminal electron acceptor (P450) are partially overlapping, which precludes the formation of a ternary protein complex^{66,67}. Considering the fusion constructs, an optimal linker should therefore facilitate the formation of an electron transfer complex between Fpr and YkuN as well as between YkuN and the P450. In view of the superior performance of the YR-P5 construct these criteria are apparently best met by the ([E/L]PPPP)₅ linker. Indeed, both the activity of Fpr (NADPH oxidation rate) and YkuN (heme-iron reduction) are reasonably well preserved in the YR-P5 construct (Tables 1 and 2). Herein, the rigid P5 linker may restrict the degrees of freedom of the fused redox partners, while allowing them to mutually interact in an effective manner, thus promoting *intramolecular* electron transfer. On the other hand, the extended rigid P5 linker (25 residues) may increase the distance between the redox centres of the fusion partners, thereby disfavours *intramolecular* electron transfer and facilitating electron transfer between fusion proteins (*i.e.* *intermolecularly*). The available experimental data for YR-P5 seems to be more consistent with a predominantly *intermolecular* electron transfer pathway. YR-P5 lacks a strict functional coupling between the Fpr and YkuN domain and its Fpr domain is readily accessible for external YkuN (Fig. 3), which is likely facilitated by a higher degree of separation of the fusion partners in case of the P5 linker. Moreover, P450 mediated catalysis exhibited a higher order (sigmoidal) dependence on fusion enzyme concentration suggesting an *intermolecular* contribution, whereas a linear dependence would have been indicative of exclusive *intramolecular* electron transfer (Fig. 1b). At elevated redox partner concentration, which increases the collision frequency between fusion proteins, apparent limitations in electron transfer were (partially) overcome and the performance of YR-P5 was similar to that of the non-fused Fpr/YkuN (Fig. 1b).

Finally, the superior construct YR-P5 exhibits versatility as it effectively supported monooxygenase activity of functionally diverse P450s, including *B. subtilis* CYP109B1 (Fig. 1), *T. fusca* YX CYP154E1 (Fig. 4), *B. megaterium* CYP106A2 (Table 3) and bovine CYP21A2 (Fig. 5). Thus, functional promiscuity of YR-P5 is not limited to bacterial P450s.

Considering the feasibility of the Fpr/YkuN system and its fused derivative YR-P5 for applied biocatalysis, the TTN presented here are rather modest. In case of CYP154E1 with β-ionone as substrate TTN were 1,582 with YR-P5 and 1,948 with the non-fused Fpr/YkuN (Supplementary Table S5). On the other hand, we recently observed that a Fpr/YkuN/CYP154E1 system can achieve TTN of up to 20,000 with stilbene as substrate⁶⁸. Although TTN in P450-based multi-component systems depend on many factors, the P450-substrate match seems to play a prominent role. For example, CYP154C5 catalysis supported by PdR/Pdx achieved TTN of 2,440 with progesterone and 3,341 with androstenedione under optimized conditions⁶⁹. For the natural fusion P450 BM3 from *Bacillus megaterium* (and mutants thereof), TTN values range from 890 with omeprazole⁷⁰, 2,200 with *n*-octane⁷¹, to 6,195 with anisole⁷², and even TTN as high as 24,363 or 45,000 have been reported in case of cyclooctane⁷³ and propane⁷⁴, respectively.

Taken together, the intrinsic dependence on redox partner(s) represents an important limiting factor in the utilization of P450s as biocatalysts. Availability of suitable redox partner(s) is therefore a prerequisite to successfully explore the catalytic potential of P450s¹⁰. By covalent fusion the complexity of the bacterial Fpr/YkuN electron transfer chain was effectively reduced, allowing easy protein production, purification and handling. Moreover, a stabilising effect on Fpr was noted upon fusion to YkuN (Supplementary Fig. S2). Through linker engineering the activity of the fusion construct could be tuned such that the high activity of the individual redox partners and the promiscuity for different P450s was largely preserved, with overall best performance of the

YR-P5 construct. The versatile YR-P5 may serve as an effective surrogate electron transfer system for exploitation of the catalytic potential of (orphan) P450s.

Methods

Generation of YkuN-Fpr fusion constructs. Construction of the *ykuN:fpr* fusion gene was performed by fusing the *Bacillus subtilis* flavodoxin gene *ykuN* lacking its stop codon to the 5'-end of the flavodoxin reductase gene *fpr* from *E. coli* JM109 by means of PCR with overlapping primers using pET16b-*ykuN*¹⁶ and pET11a-*hAR*¹⁹ as respective DNA-templates. Three extra nucleotides (GCC) were incorporated at the fusion site to create a unique *NcoI* restriction site that facilitates linker (DuaLinX) insertion. DuaLinX are double stranded DNA elements of different lengths with *NcoI*-compatible overhangs, which depending on their orientation after insertion code for either (GGGGG)_n or ([E/L]PPPP)_n linkers (n = 1–5)¹⁹. Moreover, molecular dynamics (MD) simulations were carried out on these peptide linkers to gain insight in their intrinsic structural properties. Procedures for fusion PCR, linker insertion and MD simulations are described in detail in the accompanying online Supplementary Information. The different YkuN-Fpr fusion proteins were produced in *E. coli* BL21(DE3) and purified from the cytosol via their N-terminal His₆-tag. Expression and purification procedures of the various proteins used in this study are described in the online Supplementary Information.

P450-catalysed conversion reactions. *CYP109B1* reaction mixtures contained 50 mM Tris-HCl pH 7.5, 1 mM MgCl₂, 4 mM glucose-6-phosphate (G6P), 1 U G6P-dehydrogenase from *Saccharomyces cerevisiae*, 1 μM *CYP109B1* and redox protein(s) as indicated. Stock solutions of myristic acid (10 mM, dissolved in DMSO) and NADPH (1 mM, dissolved in 50 mM Tris-HCl, pH 7.5) were pre-mixed at 1:10 ratio, respectively. Hereof, 44 μl was used to start the reactions (final concentrations: 200 μM for both myristic acid and NADPH, and 2% (v/v) DMSO). Total reaction volume was 200 μl. Reactions were allowed to proceed for 120 min at 30 °C and 300 rpm in a thermo-shaker and then stopped by the addition of HCl (8 μl, 37% w/w). For quantitative gas-liquid chromatography/mass spectrometry (GC/MS) analysis, the internal standard tridecanoic acid (dissolved in 100% DMSO) was added to a final concentration of 50 μM. For determination of NADPH consumption rates and coupling efficiencies, the *CYP109B1* reactions mixtures lacked G6P-dehydrogenase and were transferred to 96-microwell plate followed by 2 min incubation at 30 °C in a TECAN infinite m200 pro plate reader equipped with an injector module. Reactions were started by injecting 44 μl of the NADPH-myristic acid solution (described above) and the change in absorbance was measured at 340 nm at 30 °C. The NADPH consumption rate was calculated using $\epsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$. After all NADPH was consumed, HCl and internal standard were added as described above for GC/MS analysis. The employed ratio of NADPH:myristic acid was 1:1 (200 μM each, respectively) and thus the coupling efficiency equals the proportion (%) of myristic acid converted upon NADPH depletion. *CYP154E1* activity was determined in 125 μl reaction mixtures containing 100 mM KPi pH 7.5, 1 μM *CYP154E1* together with 4 μM each of Fpr and YkuN (unless stated otherwise) or 4 μM of YkuN-Fpr fusion construct, 2 mM β-ionone (dissolved in DMSO, yielding a final DMSO concentration of 2% v/v in the conversion assay) and 200 μM NADP⁺. For NADPH regeneration and removal of hydrogen peroxide, 0.63 U GDH and 20 mM glucose, and 75 U catalase from bovine liver (Sigma-Aldrich) were also included, respectively. Reactions were performed at 30 °C and 600 rpm for 1 h and then extracted by the addition of 100 μl ethyl acetate under vigorous vortexing. For quantification purposes α-ionone (internal standard) was added to a final concentration of 2 mM. *CYP106A2* reactions were performed in 250 μl total reaction volume and contained 50 mM KPi, pH 7.4, 0.5 μM *CYP106A2* together with the individual or fused redox partners at 2 or 5 μM, 200 μM of the substrate progesterone (dissolved in 100% ethanol), 5 mM G6P, 1 U G6P-dehydrogenase and 1 mM MgCl₂. The reactions were started by addition of NADPH (final concentration 100 μM) and incubated at 30 °C and 900 rpm. At different time points, the reactions were stopped by the addition of ethyl acetate. Extraction was carried out two times using 250 μl ethyl acetate each, by vigorous mixing. *CYP21A2* reactions (250 μl) contained 50 mM HEPES pH 7.4, 0.05% (v/v) Tween 20, 0.5 μM *CYP21A2*, 5 μM redox partner, 200 μM of the substrate progesterone (dissolved in 100% ethanol), 5 mM G6P, 1 U G6P-dehydrogenase and 1 mM MgCl₂. The reactions were started by addition of NADPH (final concentration 100 μM) and allowed to proceed for 30 min at 37 °C and 900 rpm. Extraction and HPLC analysis was as described for *CYP106A2*.

Quantification of substrates and product identification. Presented conversion data are average values of multiple (at least three) independent conversion reactions with indicated standard deviations. Quantification of substrates and product identification were carried out as follows. *CYP109B1* - For quantification of myristic acid (MA), the detector response was calibrated using tridecanoic acid (TDA) as internal standard. For this purpose, TDA at a fixed concentration of 50 μM was mixed with MA at final concentrations ranging from 5–200 μM in 50 mM Tris-HCl buffer, pH 7.5. Samples were then treated, extracted and analysed by GC/MS as described for normal conversion reactions. The ratio of the area of the MA to that of the TDA was plotted against the MA concentration and yielded a linear calibration curve. *CYP154E1* - For the quantification of β-ionone, a calibration curve was recorded with different concentrations of β-ionone ranging from 0.05–5 mM using 2 mM of α-ionone as internal standard. Conversion products were identified by their characteristic mass fragmentation patterns. In case of β-ionone conversion, the main conversion product 4-hydroxy-β-ionone was identified by its characteristic mass fragmentation pattern and retention time, and compared to those of the authentic reference compound. *CYP106A2/CYP21A2* - The organic phases were combined and evaporated, after which the samples were stored at –20 °C until reversed-phase HPLC analysis. Evaluation of progesterone conversion and product formation was performed by HPLC peak integration, with the total peak area of substrate plus products set to 100%. The procedures for GC/MS and HPLC analyses are described in detail in the accompanying online Supplementary Information.

Reduction of the heme-iron of CYP109B1 by various redox partners. Reduction of the heme iron was monitored by using a CO-difference spectroscopy-based method³⁴. The reduction rate of heme Fe³⁺ to Fe²⁺ was measured under anaerobic conditions (nitrogen atmosphere) in a glovebox (Glovebox Systemtechnik, Malsch, Germany) at room temperature (20 °C). Buffer and protein solutions were incubated under anaerobic conditions to obtain oxygen-free solutions. Reduction of the P450 can be determined in the presence of CO by monitoring the formation of the characteristic absorbance peak at 450 nm. Absorbance spectra (340–700 nm) were recorded using a diode array spectrophotometer (TIDAS E BASE / VIS-NIR, J&M Analytik, Essingen, Germany). To this end, CYP109B1 (2 µM) and myristic acid (400 µM) were premixed in buffer containing 50 mM Tris-HCl, pH 7.5 and flushed with 1 ml of CO gas using a gas-tight syringe. Redox partner(s) were premixed in a separate solution containing 50 mM Tris-HCl, 8 µM redox partner(s) and 2 mM NADPH. Then, 100 µl of the P450 solution were transferred to UV-cuvette and manually mixed with 100 µl of the solution containing the reduced redox partner(s). Kinetic traces were extracted from the acquired spectra by plotting the $A_{450} - A_{490}$ against the time⁷⁵. Kinetic data were analysed using OriginPro 9.0 software using a fitting routine with two-exponential steps. The equation used for the calculations was $A = A_1(1 - e^{-k_1t}) + A_2(1 - e^{-k_2t}) + C$.

Data availability. The data generated or analysed during the current study are either included in this published article (and its Supplementary Information files) or are available from the corresponding author on reasonable request.

References

- Lamb, D. C. & Waterman, M. R. Unusual properties of the cytochrome P450 superfamily. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **368**, 20120434, doi:10.1098/rstb.2012.0434 (2013).
- Girvan, H. M. & Munro, A. W. Applications of microbial cytochrome P450 enzymes in biotechnology and synthetic biology. *Curr. Opin. Chem. Biol.* **31**, 136–145, doi:10.1016/j.cbpa.2016.02.018 (2016).
- Whitehouse, C. J., Bell, S. G. & Wong, L. L. P450(BM3) (CYP102A1): connecting the dots. *Chem. Soc. Rev.* **41**, 1218–1260, doi:10.1039/c1cs15192d (2012).
- Podust, L. M. & Sherman, D. H. Diversity of P450 enzymes in the biosynthesis of natural products. *Nat. Prod. Rep.* **29**, 1251–1266, doi:10.1039/c2np20020a (2012).
- Bernhardt, R. Cytochromes P450 as versatile biocatalysts. *J. Biotechnol.* **124**, 128–145, doi:10.1016/j.jbiotec.2006.01.026 (2006).
- Behrendorff, J. B. & Gillam, E. M. Prospects for Applying Synthetic Biology to Toxicology: Future Opportunities and Current Limitations for the Repurposing of Cytochrome P450 Systems. *Chem. Res. Toxicol.* **30**, 453–468, doi:10.1021/acs.chemrestox.6b00396 (2017).
- Denisov, I. G., Makris, T. M., Sligar, S. G. & Schlichting, I. Structure and chemistry of cytochrome P450. *Chem. Rev.* **105**, 2253–2277 (2005).
- Hannemann, F., Bichet, A., Ewen, K. M. & Bernhardt, R. Cytochrome P450 systems - biological variations of electron transport chains. *Biochim. Biophys. Acta* **1770**, 330–344 (2007).
- McLean, K. J., Luciakova, D., Belcher, J., Tee, K. L. & Munro, A. W. Biological diversity of cytochrome P450 redox partner systems. *Adv. Exp. Med. Biol.* **851**, 299–317, doi:10.1007/978-3-319-16009-2_11 (2015).
- Urlacher, V. B. & Girhard, M. Cytochrome P450 monooxygenases: an update on perspectives for synthetic application. *Trends Biotechnol.* **30**, 26–36, doi:10.1016/j.tibtech.2011.06.012 (2012).
- Hlavica, P. Assembly of non-natural electron transfer conduits in the cytochrome P450 system: a critical assessment and update of artificial redox constructs amenable to exploitation in biotechnological areas. *Biotechnol. Adv.* **27**, 103–121 (2009).
- Girhard, M. *et al.* Regioselective biooxidation of (+)-valencene by recombinant *E. coli* expressing CYP109B1 from *Bacillus subtilis* in a two-liquid-phase system. *Microb. Cell Fact.* **8**, 36 (2009).
- Duan, Y. *et al.* Semi-rational engineering of cytochrome CYP153A from *Marinobacter aquaeolei* for improved omega-hydroxylation activity towards oleic acid. *Appl. Microbiol. Biotechnol.* doi:10.1007/s00253-016-7634-1 (2016).
- Ewen, K. M., Ringle, M. & Bernhardt, R. Adrenodoxin—a versatile ferredoxin. *IUBMB Life* **64**, 506–512, doi:10.1002/iub.1029 (2012).
- Khatri, Y. *et al.* Regioselective hydroxylation of norisoprenoids by CYP109D1 from *Sorangium cellulosum* So ce56. *Appl. Microbiol. Biotechnol.* **88**, 485–495, doi:10.1007/s00253-010-2756-3 (2010).
- Girhard, M., Klaus, T., Khatri, Y., Bernhardt, R. & Urlacher, V. B. Characterization of the versatile monooxygenase CYP109B1 from *Bacillus subtilis*. *Appl. Microbiol. Biotechnol.* **87**, 595–607, doi:10.1007/s00253-010-2472-z (2010).
- von Bühler, C., Le-Huu, P. & Urlacher, V. B. Cluster screening: an effective approach for probing the substrate space of uncharacterized cytochrome P450s. *ChemBioChem* **14**, 2189–2198, doi:10.1002/cbic.201300271 (2013).
- Cao, P. R., Bulow, H., Dumas, B. & Bernhardt, R. Construction and characterization of a catalytic fusion protein system: P-450(11beta)-adrenodoxin reductase-adrenodoxin. *Biochim. Biophys. Acta* **1476**, 253–264, doi:S0167-4838(99)00243-5 (2000).
- Bakkes, P. J. *et al.* Design and improvement of artificial redox modules by molecular fusion of flavodoxin and flavodoxin reductase from *Escherichia coli*. *Sci. Rep.* **5**, 12158, doi:10.1038/srep12158 (2015).
- Sadeghi, S. J. & Gilardi, G. Chimeric P450 enzymes: activity of artificial redox fusions driven by different reductases for biotechnological applications. *Biotechnol. Appl. Biochem.* **60**, 102–110 (2013).
- Hirakawa, H., Kamiya, N., Tanaka, T. & Nagamune, T. Intramolecular electron transfer in a cytochrome P450cam system with a site-specific branched structure. *Protein Eng. Des. Sel.* **20**, 453–459 (2007).
- Sadeghi, S. J., Mehareenna, Y. T., Fantuzzi, A., Valetti, F. & Gilardi, G. Engineering artificial redox chains by molecular 'Lego'. *Faraday Discuss.* **135–153**(discussion), 171–190 (2000).
- Li, S., Podust, L. M. & Sherman, D. H. Engineering and analysis of a self-sufficient biosynthetic cytochrome P450 PikC fused to the RhFRED reductase domain. *J. Am. Chem. Soc.* **129**, 12940–12941, doi:10.1021/ja075842d (2007).
- Li, S. *et al.* Selective oxidation of carboline C-H bonds by an engineered macrolide P450 mono-oxygenase. *Proc. Natl. Acad. Sci. USA* **106**, 18463–18468, doi:10.1073/pnas.0907203106 (2009).
- Shibata, M., Sakaki, T., Yabusaki, Y., Murakami, H. & Ohkawa, H. Genetically engineered P450 monooxygenases: construction of bovine P450c17/yeast reductase fused enzymes. *DNA Cell Biol.* **9**, 27–36 (1990).
- McLean, K. J., Girvan, H. M. & Munro, A. W. Cytochrome P450/redox partner fusion enzymes: biotechnological and toxicological prospects. *Expert Opin. Drug Metab. Toxicol.* **3**, 847–863, doi:10.1517/17425255.3.6.847 (2007).
- Lawson, R. J., von Wachenfeldt, C., Haq, I., Perkins, J. & Munro, A. W. Expression and characterization of the two flavodoxin proteins of *Bacillus subtilis*, YkuN and YkuP: biophysical properties and interactions with cytochrome P450 BioI. *Biochemistry* **43**, 12390–12409 (2004).
- Purdy, M. M., Koo, L. S., Ortiz de Montellano, P. R. & Klinman, J. P. Steady-state kinetic investigation of cytochrome P450cam: interaction with redox partners and reaction with molecular oxygen. *Biochemistry* **43**, 271–281 (2004).

29. Jenkins, C. M. & Waterman, M. R. NADPH-flavodoxin reductase and flavodoxin from *Escherichia coli*: characteristics as a soluble microsomal P450 reductase. *Biochemistry* **37**, 6106–6113 (1998).
30. Deng, Z. *et al.* A productive NADP⁺ binding mode of ferredoxin-NADP⁺ reductase revealed by protein engineering and crystallographic studies. *Nat. Struct. Biol.* **6**, 847–853, doi:10.1038/12307 (1999).
31. Piubelli, L. *et al.* Competition between C-terminal tyrosine and nicotinamide modulates pyridine nucleotide affinity and specificity in plant ferredoxin-NADP(+) reductase. *J. Biol. Chem.* **275**, 10472–10476 (2000).
32. Nogue, I. *et al.* Role of the C-terminal tyrosine of ferredoxin-nicotinamide adenine dinucleotide phosphate reductase in the electron transfer processes with its protein partners ferredoxin and flavodoxin. *Biochemistry* **43**, 6127–6137, doi:10.1021/bi049858h (2004).
33. Moser, C. C., Keske, J. M., Warncke, K., Farid, R. S. & Dutton, P. L. Nature of biological electron transfer. *Nature* **355**, 796–802, doi:10.1038/355796a0 (1992).
34. Omura, T. & Sato, R. The Carbon Monoxide-Binding Pigment of Liver Microsomes. I. Evidence for Its Hemoprotein Nature. *J. Biol. Chem.* **239**, 2370–2378 (1964).
35. Girhard, M., Tieves, F., Weber, E., Smit, M. S. & Ullrich, V. B. Cytochrome P450 reductase from *Candida apicola*: versatile redox partner for bacterial P450s. *Appl. Microbiol. Biotechnol.* **97**, 1625–1635, doi:10.1007/s00253-012-4026-z (2013).
36. Bogazkaya, A. M. *et al.* Selective allylic hydroxylation of acyclic terpenoids by CYP154E1 from *Thermobifida fusca* YX. *Beilstein J. Org. Chem.* **10**, 1347–1353, doi:10.3762/bjoc.10.137 (2014).
37. Brenna, E., Fuganti, C., Serra, S. & Kraft, P. Optically active ionones and derivatives: Preparation and olfactory properties. *Eur. J. Org. Chem.*, 967–978 (2002).
38. Lalko, J. *et al.* Fragrance material review on beta-ionone. *Food Chem. Toxicol.* **45**(Suppl 1), S241–247, doi:10.1016/j.fct.2007.09.052 (2007).
39. Kakeya, H., Sugai, T. & Ohta, H. Biochemical preparation of optically active 4-hydroxy- β -ionone and its transformation to (S)-6-hydroxy- α -ionone. *Agric. Biol. Chem.* **55**, 1873–1876 (1991).
40. Eschenmoser, W., Uebelhart, P. & Eugster, C. H. Synthesis and Chirality of the Enantiomeric 6-Hydroxy- α -Ionones and of Cis and Trans-5,6-Dihydroxy-5,6-Dihydro-Beta-Ionones. *Helv. Chim. Acta* **64**, 2681–2690 (1981).
41. Ullrich, V. B., Makhsumkhanov, A. & Schmid, R. D. Biotransformation of beta-ionone by engineered cytochrome P450 BM-3. *Appl. Microbiol. Biotechnol.* **70**, 53–59 (2006).
42. Berg, A., Gustafsson, J. A. & Ingelman-Sundberg, M. Characterization of a cytochrome P-450-dependent steroid hydroxylase system present in *Bacillus megaterium*. *J. Biol. Chem.* **251**, 2831–2838 (1976).
43. Lisurek, M., Simgen, B., Antes, I. & Bernhardt, R. Theoretical and experimental evaluation of a CYP106A2 low homology model and production of mutants with changed activity and selectivity of hydroxylation. *ChemBioChem* **9**, 1439–1449, doi:10.1002/cbic.200700670 (2008).
44. Schmitz, D., Zapp, J. & Bernhardt, R. Steroid conversion with CYP106A2 - production of pharmaceutically interesting DHEA metabolites. *Microb. Cell Fact.* **13**, 81, doi:10.1186/1475-2859-13-81 (2014).
45. Bleif, S. *et al.* A new *Bacillus megaterium* whole-cell catalyst for the hydroxylation of the pentacyclic triterpene 11-keto-beta-sowwellic acid (KBA) based on a recombinant cytochrome P450 system. *Appl. Microbiol. Biotechnol.* **93**, 1135–1146, doi:10.1007/s00253-011-3467-0 (2012).
46. White, P. C., Tusie-Luna, M. T., New, M. I. & Speiser, P. W. Mutations in steroid 21-hydroxylase (CYP21). *Hum. Mutat.* **3**, 373–378, doi:10.1002/humu.1380030408 (1994).
47. Brixius-Anderko, S., Schiffer, L., Hannemann, F., Janocha, B. & Bernhardt, R. A CYP21A2 based whole-cell system in *Escherichia coli* for the biotechnological production of premedrol. *Microb. Cell Fact.* **14**, 135, doi:10.1186/s12934-015-0333-2 (2015).
48. Blaschkowski, H. P., Neuer, G., Ludwig-Festl, M. & Knappe, J. Routes of flavodoxin and ferredoxin reduction in *Escherichia coli*. CoA-acylating pyruvate: flavodoxin and NADPH: flavodoxin oxidoreductases participating in the activation of pyruvate formate-lyase. *Eur. J. Biochem.* **123**, 563–569 (1982).
49. Bianchi, V. *et al.* Flavodoxin is required for the activation of the anaerobic ribonucleotide reductase. *Biochem. Biophys. Res. Commun.* **197**, 792–797, doi:10.1006/bbrc.1993.2548 (1993).
50. Razquin, P. *et al.* Differential activities of heterocyst ferredoxin, vegetative cell ferredoxin, and flavodoxin as electron carriers in nitrogen fixation and photosynthesis in *Anabaena* sp. *Photosynth. Res.* **43**, 35–40, doi:10.1007/BF00029460 (1995).
51. Nieva-Gomez, D., Roberts, G. P., Kleivickis, S. & Brill, W. J. Electron transport to nitrogenase in *Klebsiella pneumoniae*. *Proc. Natl. Acad. Sci. U. S. A.* **77**, 2555–2558 (1980).
52. Fujii, K. & Huennekens, F. M. Activation of methionine synthetase by a reduced triphosphopyridine nucleotide-dependent flavoprotein system. *J. Biol. Chem.* **249**, 6745–6753 (1974).
53. Birch, O. M., Fuhrmann, M. & Shaw, N. M. Biotin synthase from *Escherichia coli*, an investigation of the low molecular weight and protein components required for activity *in vitro*. *J. Biol. Chem.* **270**, 19158–19165 (1995).
54. Dong, M. S., Yamazaki, H., Guo, Z. & Guengerich, F. P. Recombinant human cytochrome P450 1A2 and an N-terminal-truncated form: construction, purification, aggregation properties, and interactions with flavodoxin, ferredoxin, and NADPH-cytochrome P450 reductase. *Arch. Biochem. Biophys.* **327**, 11–19, doi:10.1006/abbi.1996.0086 (1996).
55. McIver, L. *et al.* Characterisation of flavodoxin NADP⁺ oxidoreductase and flavodoxin; key components of electron transfer in *Escherichia coli*. *Eur. J. Biochem.* **257**, 577–585 (1998).
56. Sabbadin, F., Grogan, G. & Bruce, N. C. LICRED: a versatile drop-in vector for rapid generation of redox-self-sufficient cytochromes P450. *Methods Mol. Biol.* **987**, 239–249, doi:10.1007/978-1-62703-321-3_20 (2013).
57. Hirakawa, H. & Nagamune, T. Use of *Sulfolobus solfataricus* PCNA subunit proteins to direct the assembly of multimeric enzyme complexes. *Methods Mol. Biol.* **978**, 149–163, doi:10.1007/978-1-62703-293-3_11 (2013).
58. Jung, S. T., Lauchli, R. & Arnold, F. H. Cytochrome P450: taming a wild type enzyme. *Curr. Opin. Biotechnol.* **22**, 809–817, doi:10.1016/j.copbio.2011.02.008 (2011).
59. Argos, P. An investigation of oligopeptides linking domains in protein tertiary structures and possible candidates for general gene fusion. *J. Mol. Biol.* **211**, 943–958, doi:10.1016/0022-2836(90)90085-Z (1990).
60. George, R. A. & Heringa, J. An analysis of protein domain linkers: their classification and role in protein folding. *Protein Eng.* **15**, 871–879 (2002).
61. Tanaka, T., Yokoyama, S. & Kuroda, Y. Improvement of domain linker prediction by incorporating loop-length-dependent characteristics. *Biopolymers* **84**, 161–168, doi:10.1002/bip.20361 (2006).
62. Chichili, V. P. R., Kumar, V. & Sivaraman, J. Linkers in the structural biology of protein-protein interactions. *Protein Sci.* **22**, 153–167, doi:10.1002/pro.2206 (2013).
63. Evers, T. H., van Dongen, E. M., Faesen, A. C., Meijer, E. W. & Merckx, M. Quantitative understanding of the energy transfer between fluorescent proteins connected via flexible peptide linkers. *Biochemistry* **45**, 13183–13192, doi:10.1021/bi061288t (2006).
64. Arai, R., Wriggers, W., Nishikawa, Y., Nagamune, T. & Fujisawa, T. Conformations of variably linked chimeric proteins evaluated by synchrotron X-ray small-angle scattering. *Proteins* **57**, 829–838, doi:10.1002/prot.20244 (2004).
65. Klein, J. S., Jiang, S., Galimidi, R. P., Keefe, J. R. & Bjorkman, P. J. Design and characterization of structured protein linkers with differing flexibilities. *Protein Eng. Des. Sel.* **27**, 325–330, doi:10.1093/protein/gzu043 (2014).
66. Hall, D. A. *et al.* Mapping the interactions between flavodoxin and its physiological partners flavodoxin reductase and cobalamin-dependent methionine synthase. *Proc. Natl. Acad. Sci. USA* **98**, 9521–9526 (2001).

67. Hlavica, P. Mechanistic basis of electron transfer to cytochromes p450 by natural redox partners and artificial donor constructs. *Adv. Exp. Med. Biol.* **851**, 247–297, doi:10.1007/978-3-319-16009-2_10 (2015).
68. Rühlmann, A., Antovic, D., Müller, T. J. J. & Urlacher, V. B. Regioselective Hydroxylation of Stilbenes by Engineered Cytochrome P450 from *Thermobifida fusca* YX. *Adv. Synth. Catal.* **359**, 984–994 (2017).
69. Bracco, P., Janssen, D. B. & Schallmeyer, A. Selective steroid oxyfunctionalisation by CYP154C5, a bacterial cytochrome P450. *Microb. Cell Fact.* **12**, 95, doi:10.1186/1475-2859-12-95 (2013).
70. Jang, H. H. *et al.* Regioselective C–H hydroxylation of omeprazole sulfide by *Bacillus megaterium* CYP102A1 to produce a human metabolite. *Biotechnol. Lett.* **39**, 105–112, doi:10.1007/s10529-016-2211-3 (2017).
71. Maurer, S. C. *et al.* Catalytic hydroxylation in biphasic systems using CYP102A1 mutants. *Adv. Synth. Catal.* **347**, 1090–1098 (2005).
72. Dennig, A., Lülldorf, N., Liu, H. & Schwaneberg, U. Regioselective o-hydroxylation of monosubstituted benzenes by P450 BM3. *Angew. Chem. Int. Ed. Engl.* **52**, 8459–8462, doi:10.1002/anie.201303986 (2013).
73. Müller, C. A. *et al.* A whole cell biocatalyst for double oxidation of cyclooctane. *J. Ind. Microbiol. Biotechnol.* **43**, 1641–1646, doi:10.1007/s10295-016-1844-5 (2016).
74. Fasan, R., Chen, M. M., Crook, N. C. & Arnold, F. H. Engineered alkane-hydroxylating cytochrome P450(BM3) exhibiting natively catalytic properties. *Angew. Chem. Int. Ed. Engl.* **46**, 8414–8418 (2007).
75. Honda Malca, S., Girhard, M., Schuster, S., Durre, P. & Urlacher, V. B. Expression, purification and characterization of two *Clostridium acetobutylicum* flavodoxins: Potential electron transfer partners for CYP152A2. *Biochim. Biophys. Acta* **1814**, 257–264, doi:10.1016/j.bbapap.2010.06.013 (2011).
76. Lisurek, M., Kang, M. J., Hartmann, R. W. & Bernhardt, R. Identification of monohydroxy progesterones produced by CYP106A2 using comparative HPLC and electrospray ionisation collision-induced dissociation mass spectrometry. *Biochem. Biophys. Res. Commun.* **319**, 677–682 (2004).

Acknowledgements

Simone Brixius-Anderko kindly provided the purified CYP21A2. This work was generously supported by the German Federal Ministry of Education and Research (BMBF), grant FKZ 031A166.

Author Contributions

P.J.B. conceived and conducted most of the experiments, analysed and interpreted the data associated with the experiments, designed the artwork, and is the main author of the manuscript. T.S. and A.R. contributed to conversion experiments with different P450s. J.R. and M.C.H. conducted and evaluated M.D. simulation experiments. P.S. and S.B. contributed to initial cloning and expression of the different redox constructs. M.G., R.B. and V.B.U. contributed to conceiving experiments, interpretation of data and writing of the manuscript. All authors reviewed and gave approval to the final version of the manuscript.

Additional Information

Supplementary information accompanies this paper at doi:10.1038/s41598-017-10075-w

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

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2017

Supplementary Information

to

**Engineering of versatile redox partner fusion enzymes that support
monooxygenase activity of functionally diverse cytochrome P450s**

*Patrick J. Bakkes¹, Jan L. Riehm², Tanja Sagadin³, Ansgar Rühlmann¹, Peter Schubert¹, Stefan Biemann¹,
Marco Girhard¹, Michael C. Hutter², Rita Bernhardt³ and Vlada B. Urlacher^{1*}*

¹ Institute of Biochemistry, Heinrich Heine University Düsseldorf, Universitätsstr. 1, 40225 Düsseldorf, Germany

² Center for Bioinformatics, Saarland University, Campus Building E2.1, 66123 Saarbrücken, Germany

³ Institute of Biochemistry Saarland University, Campus Building B2.2, 66123 Saarbrücken, Germany

* Corresponding author: Vlada B. Urlacher (vlada.urlacher@uni-duesseldorf.de)

METHODS

Bacterial strains, commercial enzymes and chemicals - *E. coli* DH5 α (Invitrogen, Karlsruhe, Germany) was used for molecular cloning and *E. coli* BL21(DE3) (Novagen, Darmstadt, Germany) was used for recombinant gene expression. Bacteria were grown in LB medium containing 100 μ g/ml ampicillin. Molecular cloning and microbiological procedures were carried out by standard procedures¹. Oligonucleotides were obtained from Eurofins MWG Operon (Ebersberg, Germany). Phusion DNA polymerase, Restriction enzymes, FastAP thermosensitive alkaline phosphatase, T4 DNA ligase and isopropyl- β -D-thiogalactopyranoside (IPTG) were obtained from Thermo Fisher Scientific (St. Leon-Rot, Germany). Glucose-6-phosphate dehydrogenase from *Saccharomyces cerevisiae* was purchased from Roche Diagnostics (Mannheim, Germany). NADPH was from Carbosynth (Berkshire, UK), whereas all other chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany).

Construction of the *ykuN:fpr* fusion gene - The primers used for the construction of *ykuN:fpr* are listed in Table S1. First *ykuN* and *fpr* were amplified separately using primer pairs 1-2 and 3-4, respectively (Table S1). Primer 1 introduced an N-terminal His₆-tag and simultaneously eliminated an internal *NdeI* restriction site at the 5' end of *ykuN* via a silent point mutation (T $\rightarrow$ C). The obtained PCR products were gel purified, mixed in a 1:1 ratio, and then subjected to a second round of PCR in the absence of additional primers. After 6 cycles, primers 1 and 4 were added to the mixture and PCR was allowed to continue for an additional 30 cycles. The *ykuN:fpr* fusion gene was then gel purified and subsequently cloned into pET11a (Novagen) using *NdeI* and *BamHI*, yielding pET11a-*hYR*. DNA sequencing (GATC-Biotech, Konstanz, Germany) confirmed that the sequence of *ykuN:fpr* was correct.

Supplementary Table S1. PCR primers used to fuse *ykuN* to *fpr*. Underlined nucleotides indicate restriction sites relevant to cloning; *NdeI* (CATATG) and *BamHI* (GGATCC), and linker insertion; *NcoI* (CCATGG). The overlapping primer pair 2-3 are fully complementary and lack the *ykuN* stop codon, which allows the in frame fusion of *ykuN* to *fpr*, with nucleotides of *fpr* highlighted in grey. To generate the *NcoI* site for *DuaLinX* insertion three extra nucleotides coding for Ala were incorporated at the fusion site of *ykuN:fpr*. As a result the DNA at the fusion site of the *ykuN:fpr* (YR) construct codes for -Ala-Met-Ala-, with minimal disturbance of the natural amino acid sequences.

Primer (name)	Nucleotide sequence
1 (<i>ykuN</i> -Fw)	5'-AGGAATTCCATATG <u>CATCATCATCATCATCAT</u> GCTAAAGCCTTGATTACATACGCCAG-3'
2 (<i>ykuN:fpr</i> -Rv)	5'-CTGTTACCCAATCAGCCATGGCTGAAACATGGATTTTTCCT-3'
3 (<i>ykuN:fpr</i> -Fw)	5'-AGGAAAAATCCATGTTTCAGCCATGGCTGATTGGGTAACAG-3'
4 (<i>fpr</i> -Rv)	5'-CAGCCGGATCCTTACCAGTAATGCTCC-3'

Linker insertion, ligation and transformation - Linker insertion between the fusion partners was carried out using the DuaLinX procedure as described in ². The DuaLinX are double stranded DNA elements of different lengths with *NcoI*-compatible overhangs, which depending on their orientation after insertion code for either flexible (GGGGS)_n or rigid ([E/L]PPPP)_n linkers. Template pET11a-*hYR* was linearized with *NcoI*, dephosphorylated and mixed with desired DuaLinX at a 1:3 molar ratio. Ligation and transformation was carried out as described in ². The obtained constructs were verified by DNA sequencing (GATC, Konstanz, Germany). In such manner a set of 10 fusion constructs harbouring linkers of 5, 10, 15, 20 and 25 residues was created (Table S2). To study expression, the different fusion constructs were used for transformation of chemi-competent *E. coli* BL21(DE3) cells.

Table S2. Linkers used to connect YkuN and Fpr using the DuaLinX tool ².

Linker	Length	Amino acid sequence
G1	5	GGGGS
P1	5	EPPPP
G2	10	GGGGS-GGGGS
P2	10	EPPPP-LPPPP
G3	15	GGGGS-GGGGS-GGGGS
P3	15	EPPPP-EPPPP-LPPPP
G4	20	GGGGS-GGGGS-GGGGS-GGGGS
P4	20	EPPPP-LPPPP-LPPPP-EPPPP
G5	25	GGGGS-GGGGS-GGGGS-GGGGS-GGGGS
P5	25	EPPPP-EPPPP-LPPPP-LPPPP-LPPPP

Expression and purification of YkuN-Fpr fusion proteins - Cryo-cultures of *E. coli* BL21(DE3) cells carrying the different fusion constructs were used to inoculate LB_{amp} medium at an OD₆₀₀ of 0.12. Cultures were grown till an OD₆₀₀ ~0.6 at 25°C and 140 rpm. Expression was then induced by the addition of IPTG (0.1 mM) and carried out for 20 h. The cells were subsequently harvested by centrifugation (8,000x g, 15 min, 4°C). After removal of the supernatant, the cell pellets were suspended in 8 ml buffer containing Tris-HCl, pH 7.5 (50 mM) and PMSF (0.1 mM). Cells were lysed by sonication on ice applying four cycles of 1 min sonication followed by 1 min intermission and the lysate was cleared by centrifugation (20,000x g, 30 min, 4°C). The supernatant was supplemented with FAD (10 µM) and FMN (10 µM) and incubated on ice for 1 h. To 4 volumes of this protein sample, 1 volume of 5x IMAC buffer containing Tris-HCl, pH 7.5 (250 mM) and NaCl (750 mM) was added and filtered through a 0.45 µm filter. The filtered protein samples were then applied to self-cast Ni²⁺-NTA (Qiagen, Hilden, Germany) columns with

a 2 ml bed volume that were equilibrated with 1x IMAC buffer, which contains Tris-HCl, pH 7.5 (50 mM) and NaCl (150 mM). Non-specifically bound proteins were removed by using 1x IMAC buffer supplemented with 10 mM imidazole, while subsequent elution of the fusion proteins was carried out with 1x IMAC buffer supplemented with 150 mM imidazole. Immediately after elution the elution fractions (~8 ml) were 1:1 diluted with 1x IMAC buffer and then concentrated to a volume of ~1 ml using centrifugal devices (Sartorius, Vivaspin 15, 30 kDa MWCO). Residual imidazole was removed by using gravity flow PD midiTrap G-25 columns (GE-Healthcare) equilibrated in 1x IMAC buffer supplemented with 5% (v/v) glycerol. Finally, the purified fusion proteins were concentrated as described above and stored at -20°C.

Expression and purification of Fpr and FldA from *E. coli* were conducted as described in ² and procedures for YkuN and CYP109B1 from *B. subtilis* were carried out as described in ³ and ⁴, respectively. *Thermobifida fusca* YX CYP154E1, *B. megaterium* CYP106A2 and bovine CYP21A2 were expressed and purified according to ⁵, ⁶ and ⁷ respectively, whereas bovine AdR and Adx were isolated as described in ⁸ and ⁹. Crystal structures of CYP109B1 ¹⁰, CYP106A2 ¹¹, CYP21A2 ¹² have recently been reported.

Determination of protein concentration - The concentration of purified P450 monooxygenases was determined using the CO-difference spectral assay with $\epsilon_{450-490} = 91 \text{ mM}^{-1}\text{cm}^{-1}$ ¹³. The concentrations of Fpr, FldA and YkuN were calculated from their respective molar extinction coefficients $\epsilon_{456} = 7,100 \text{ M}^{-1}\text{cm}^{-1}$ ¹⁴, $\epsilon_{465} = 8,420 \text{ M}^{-1}\text{cm}^{-1}$ ¹⁵ and $\epsilon_{461} = 10,000 \text{ M}^{-1}\text{cm}^{-1}$ ¹⁶. For the concentration determination of the fusion proteins a similar approach as described in ² was followed. In brief, from absorbance spectra of known concentrations of YkuN, the ϵ_{456} was determined as $9,810 \text{ M}^{-1}\text{cm}^{-1}$. The extinction coefficient of the different YkuN-Fpr fusion constructs was then calculated by addition of the ϵ_{456} of Fpr and YkuN, yielding $\epsilon_{456} = 16,910 \text{ M}^{-1}\text{cm}^{-1}$ for the fusion proteins. Typical absorbance spectra are shown in Supplementary Figs. S2 and S3. Alternatively, the total protein concentration was determined by a Bradford-based assay ¹⁷ using bovine serum albumin as a standard.

GC-MS and HPLC analysis - CYP109B1 reaction mixtures were extracted two times with 0.8 ml diethyl ether and dried by the addition of anhydrous MgSO_4 . The dried organic phase was evaporated and the residue was dissolved in 80 μl *N,O*-bis(trimethylsilyl)trifluoroacetamide containing 1% (v/v) trimethylchlorosilane for derivatization. Samples were transferred into GC vials and incubated at 80°C for 30 min. Myristic acid conversion was analyzed on a GC/MS-QP2010 (Shimadzu, Duisburg, Germany) equipped with a FS-Supreme-5 column (30 m x 0.25 mm x 0.25 μm , CS-Chromatographie Service GmbH, Langerwehe, Germany) as described previously ¹⁸. CYP154E1 - After phase separation by centrifugation, the organic phase was analyzed for β -ionone conversion on the same GC-MS system as described above.

Samples were injected at a temperature of 250°C. The column temperature was set to 130°C, which was held for 2 min and then increased to 240°C at 20°C min⁻¹. Temperature was maintained for another 2 min and then further increased to 300°C at 50°C min⁻¹ and held for 1 min. CYP106A2/CYP21A2 – Prior to HPLC analysis samples were dissolved in 200 µl of 10% (v/v) acetonitrile. HPLC experiments were performed on a Jasco system containing a PU-2080Plus Intelligent HPLC Pump, an AS-2050Plus Intelligent Sampler, an UV-2075Plus Intelligent UV/Vis Detector, a LG-2080-02 Ternary Gradient Unit, a DG-2080-53 3-Line Degasser, and a LC-NetII/ADC (Jasco, Gross-Umstadt, Germany). The HPLC system was equipped with a reversed phase ec MN Nucleodor C18 (5 µm, 4.0 x 125 mm) column (Macherey-Nagel, Düren, Germany) maintained at 40°C by a column thermostat (W.O. electronics, Austria). A mixture of acetonitrile and water (10:90) served as mobile phase, while flow rate was set to 1 ml/min. Compound separation was accomplished by mixing solvent A (water containing 10 % (v/v) acetonitrile) with solvent B (100 % acetonitrile). First a linear gradient from 11 % solvent B (and 89 % solvent A) to 33 % solvent B within 2 min was applied and then held at 33% for 4 min. Hereafter, the concentration of solvent B was increased to 100 % in 4 min and then kept at 100 % for 2 min. Finally, the column was re-equilibrated by employing a linear gradient to 11 % solvent B within 0.1 min, which was maintained for 3 min. For each measurement, 10 µL of sample was injected and absorbance was monitored at 240 nm.

Molecular dynamics (MD) simulations - To gain insight in the structural properties of the peptide linkers used for joining the redox partners in a physiological medium, molecular dynamics simulations were carried out in water as explicit solvent. Three-dimensional models for the glycine-rich (G1 - G5) and proline-rich (P1 - P5) linkers were generated using HYPERCHEM (version 6.02, Hypercube Inc., Gainsville, FL, 1999). The initial conformation of the G-linkers corresponded to a beta-sheet conformation, whereas the P-linkers were modelled as PP-helix II ($\varphi = -75^\circ$, $\psi = 150^\circ$). These initial conformations were chosen in order to start with the maximum possible length of the linkers, while simultaneously possessing an ordered structure that corresponds to an energetic minimum. Moreover, this approach allows monitoring changes in linker length during the simulation by inspecting the corresponding radius of gyration. The ends of the linkers were capped with acetyl (ACE) residues at the N-terminus and N-methylamine (NME) residues at the C-terminus. Each linker molecule was placed into a cubic box (with a scale to match the linker's length plus an additional 2 nm) of TIP3P (transferable intermolecular potential 3 point) waters¹⁹ followed by a steepest descent energy minimization that was stopped at a gradient norm lower than 1000 kJ mol⁻¹ nm⁻². Prior to the actual molecular dynamics simulations we equilibrated the linker sequences in two steps. The first step was carried out in a NVT (constant number of particles, volume and temperature) ensemble for 100 ps maintaining the temperature at 300 K using a modified Berendsen thermostat²⁰. In the second step, the pressure of the system was

stabilized at 1 bar applying a Parinello-Rahman barostat ²¹ for 100 ps of NPT (constant number of particles, pressure and temperature) equilibration. All bonds in both steps were constrained by the LINCS ²² algorithm. Subsequently, regular MD production runs were carried out for 20 ns for each linker construct. Hereby Newton's equations of motion were integrated by the leapfrog algorithm at time steps of 2 fs. Subsequent analysis included investigation of plots of the radius of gyration (Rg) for the different linker types and lengths. Rg was calculated by the root mean square distance of atoms in the linker molecule from the center of mass.

RESULTS

1. Expression and purification of the redox fusion enzymes.

Expression and purification of the fusion proteins were carried out as described above. After IMAC and removal of imidazole, the fusion proteins were analysed by 12.5% SDS-PAGE (Fig. S1).

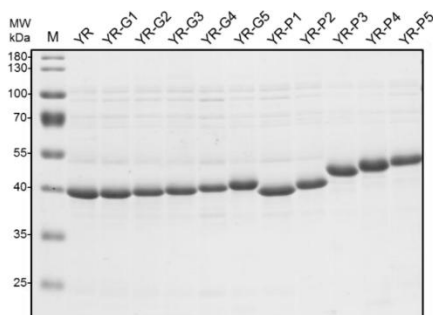


Figure S1. SDS-PAGE analysis of IMAC purified YkuN-Fpr fusion proteins. Fusion protein (2 μ g) was loaded in each lane. Indicated are YkuN-Fpr fusion construct without linker (YR) and constructs carrying flexible glycine-rich linkers G1 - G5 or rigid proline-rich linkers P1 - P5 (for linker specifications see Table S2²).

2. Spectroscopic analysis of the fusion proteins: increased stability of Fpr upon fusion to YkuN.

Protein stability of the P450s and dedicated redox partner(s) can be limiting in P450-catalyzed reactions. Previous experiments have shown that protein stability of YkuN/Fpr in CYP154A8-catalyzed production of chiral 2-alcohols is a limiting factor especially in prolonged (~18 h) conversion reactions²³. Moreover, pioneering work involving the biochemical characterization of *E. coli* Fpr revealed that this protein slowly precipitates during spectral analysis¹⁴. Consistent with this observation, we noted that purified Fpr forms visible aggregates upon dilution into buffer (50 mM Tris-HCl pH 7.5), as illustrated by the overall increase in absorbance over time due to light scatter (Fig. S2B). In contrast, under the same conditions, both YkuN and the YkuN-Fpr fusion constructs exhibited no visible aggregation and the absorbance spectra remained virtually unchanged over time (Fig. S2A and C and data not shown). Thus in the absence of stabilizing agents (such as NaCl, glycerol or interacting protein partners) Fpr is prone to aggregation, whereas Fpr is apparently stabilized upon fusion to YkuN. Moreover, even after prolonged incubation (24 h) at room temperature the characteristic absorbance maximum at 460 nm (Fig. S3) of the fusion constructs remained virtually unchanged, with no evidence of protein precipitation.

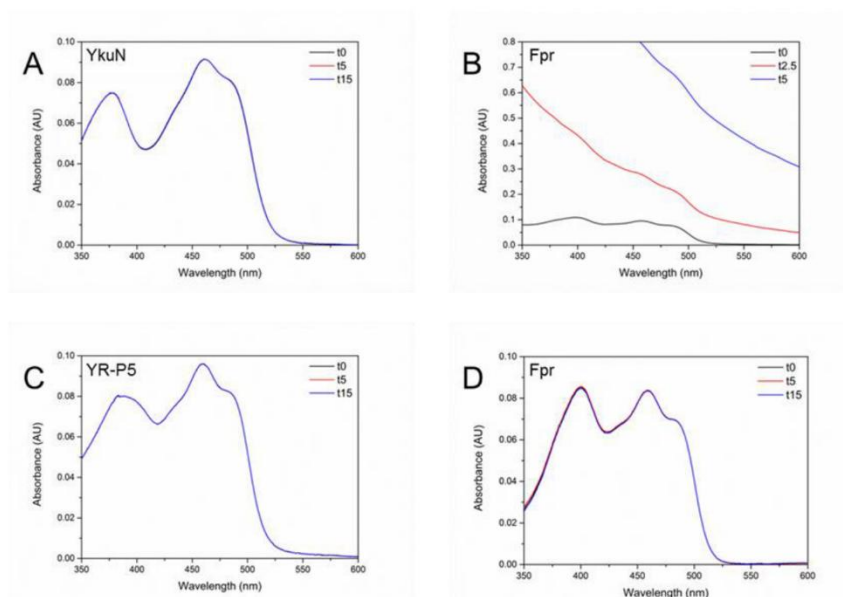


Figure S2. Stabilisation of Fpr upon fusion to YkuN. Arbitrary amounts of indicated purified redox protein were diluted in appropriate buffer (**A - C**: 50 mM Tris-HCl pH 7.5, and **D**: 50 mM KPi pH 7.5, 500 mM NaCl, 20% v/v glycerol), typically yielding final concentrations in the range of 4 - 8 μ M. At indicated time points (minutes after dilution), spectra were recorded at 25°C using a thermostated Perkin Elmer lambda 35 spectrometer. Spectra were corrected for buffer absorbance.

3. Spectral properties of the YkuN-Fpr fusion constructs.

Consistent with previous observations for fully oxidized (quinone) FMN-containing YkuN²⁴, the absorbance spectrum of purified YkuN shows two major absorbance bands with maxima at 378 nm and 462 nm, with a shoulder around 480 nm (Fig. S3A). The spectral properties of purified (oxidized) FAD-containing Fpr are nearly identical to those reported previously^{15,25}, exhibiting characteristic peaks at 400 nm and ~458 nm, with a shoulder around 480 nm (Fig. S3A). The peak at 400 nm is a rather uncommon feature for flavoproteins, which typically show a maximum at ~380 nm. This unusual absorbance property of Fpr is thought to arise from a bent conformation of the FAD cofactor governed, in part, by the extra C-terminal Trp248²⁶. The linker-less YkuN-Fpr (YR) fusion construct exhibits the hallmarks of the individual redox partners combined. The absorbance spectrum of YR shows a rather broad peak between 378 – 400 nm, with a maximum around 389 nm, and a second maximum at 460 nm, with a shoulder around 480 nm (Fig. S3A). The depicted YkuN-Fpr fusion constructs carrying different linkers showed nearly identical absorbance spectra (Fig. S3B), which indicates that the fusion constructs have a similar cofactor content (FAD + FMN) and a similar protein environment of the cofactors.

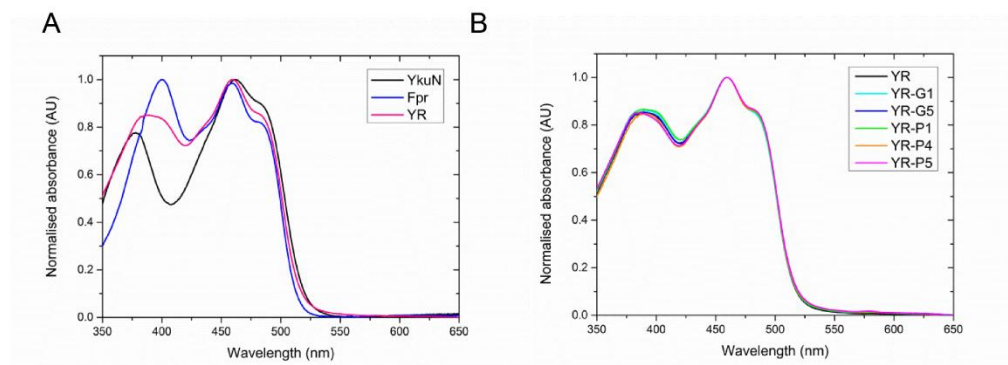


Figure S3. Spectral properties of the individual and fused redox partners, YkuN and Fpr. Arbitrary amounts of indicated redox protein were diluted in appropriate buffer (**A**, 50mM KPi pH 7.5, 500 mM NaCl, 20% (v/v) glycerol, and **B**, 50 mM Tris-HCl pH 7.5), typically yielding final concentrations in the range of 4 – 8 μ M. Spectra were recorded at 25°C using a thermostated Perkin Elmer Lambda 35 spectrometer. Presented normalised spectra are the average of 3 recorded spectra that were corrected for buffer absorbance.

4. Reduction of the CYP109B1 heme-iron by various redox partners.

Reduction of the P450 heme-iron (Fe^{3+} to Fe^{2+}) was monitored by using a CO-difference spectroscopy-based method¹³. Heme-iron reduction was measured in the presence of carbon monoxide under anaerobic conditions at 20°C, using 1 μ M CYP109B1 and 4 μ M of indicated redox partner(s). Reactions were carried out as described in the Methods section of the main paper. Kinetic traces were obtained from the acquired absorbance spectra (340 - 700 nm), by plotting the $\Delta A_{450} - A_{490}$ against the time²⁷. Kinetic data were fitted using equation $A = A_1(1 - e^{-k_1 t}) + A_2(1 - e^{-k_2 t}) + C$. Typical kinetic traces and corresponding fits (thin lines) are shown in Fig. S4.

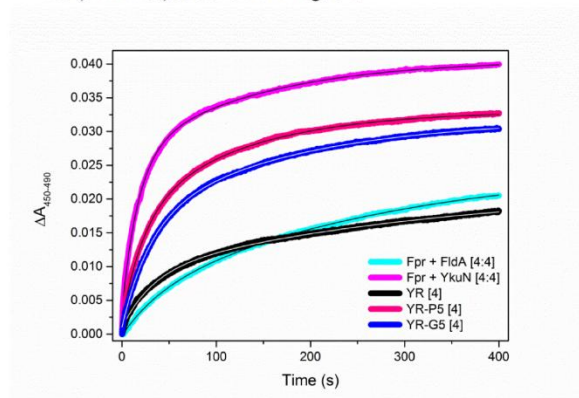


Figure S4. CYP109B1 heme-iron reduction by different redox partners.

5. Conversion of myristic acid by *B. subtilis* CYP109B1. Activity of the fatty acid hydroxylase CYP109B1¹⁰ was determined in 200 μ l reaction mixtures containing 50 mM Tris-HCl pH 7.5, 1 mM MgCl₂, 4 mM glucose-6-phosphate (G6P), 1 U *S. cerevisiae* G6P-dehydrogenase, 1 μ M CYP109B1 and redox protein(s) as indicated (Supplementary Table S3)

Supplementary Table S3. Product distribution for the CYP109B1-catalysed conversion of myristic acid in the presence of different redox partners. Myristic acid conversion data represent average values of at least three independent conversion reactions with indicated standard deviation. ¹ Values in brackets indicate final concentrations in μ M of the corresponding redox proteins. ² In CYP109B1 reactions exhibiting high turnover ($\geq 60\%$ myristic acid conversion) an unidentified product appeared (retention time 9.50 min), which likely represents an overoxidation product of myristic acid.

Redox protein(s) ¹	Myristic acid conversion (%)	Myristic acid product distribution (%)					
		ω_1	ω_2	ω_3	ω_4	ω_5	X ²
		10.37 min	10.22 min	9.97 min	9.80 min	10.72 min	9.50 min
Fpr + FldA [1:1]	22.1 $\pm$ 4.2	16.5 $\pm$ 1.2	29.7 $\pm$ 1.0	27.8 $\pm$ 0.8	18.4 $\pm$ 1.0	7.6 $\pm$ 1.5	N.D.
Fpr + YkuN [1:1]	59.9 $\pm$ 12.5	19.0 $\pm$ 0.6	31.7 $\pm$ 0.6	28.1 $\pm$ 0.7	15.8 $\pm$ 0.3	4.6 $\pm$ 0.6	0.9 $\pm$ 0.5
Fpr + YkuN [1:10]	96.0 $\pm$ 0.5	14.0 $\pm$ 1.0	26.2 $\pm$ 0.5	25.5 $\pm$ 0.2	19.6 $\pm$ 0.5	5.4 $\pm$ 0.9	9.2 $\pm$ 1.6
Fpr + YkuN [4:4]	93.5 $\pm$ 1.8	17.6 $\pm$ 0.4	29.3 $\pm$ 0.5	27.1 $\pm$ 0.4	17.8 $\pm$ 0.2	4.2 $\pm$ 0.9	3.9 $\pm$ 0.9
YR [4]	37.5 $\pm$ 2.7	18.5 $\pm$ 0.5	31.2 $\pm$ 1.2	27.8 $\pm$ 0.7	15.4 $\pm$ 0.3	6.1 $\pm$ 2.3	N.D.
YR-G5 [4]	63.0 $\pm$ 4.9	19.4 $\pm$ 0.2	32.1 $\pm$ 0.3	28.1 $\pm$ 0.4	15.6 $\pm$ 0.3	3.8 $\pm$ 1.5	1.0 $\pm$ 0.1
YR-P5 [4]	86.1 $\pm$ 2.2	19.2 $\pm$ 0.2	30.7 $\pm$ 0.2	27.4 $\pm$ 0.3	16.1 $\pm$ 0.4	4.6 $\pm$ 0.8	2.0 $\pm$ 0.3

Supplementary Table S4. Coupling efficiency for CYP154E1-mediated conversion of β -ionone using different redox partners.

Reconstituted system	Respective stoichiometry (μ M)	Coupling efficiency (%)
Fpr/YkuN/CYP154E1	1 : 10 : 1	16.7
	1 : 4 : 1	42.0
	4 : 4 : 1	44.2
YR-P5/CYP154E1	4 : 1	50.2
	1 : 1	44.8

Supplementary Table S5. Total turnover numbers for β -ionone achieved with CYP154E1 reconstituted systems.

Reconstituted system	Respective stoichiometry (μ M)	Conversion (h)	TTN
Fpr/YkuN/CYP154E1	4 : 4 : 1	1	1439
	2 : 2 : 0.5	6	1948
YR-P5/CYP154E1	4 : 4 : 1	1	976
	2 : 2 : 0.5	6	1582

6. Conversion of myristic acid by *B. subtilis* CYP109B1 in the absence of NADPH regeneration

Activity of the fatty acid hydroxylase CYP109B1 was determined as described in Methods section of the main manuscript. Reaction mixtures (200 μ l) in 96-microwell plate contained 50 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 4 mM glucose-6-phosphate (G6P), 1 μ M CYP109B1, 4 μ M redox protein(s) as indicated (Fig. S5), 200 μ M myristic acid and 200 μ M NADPH.

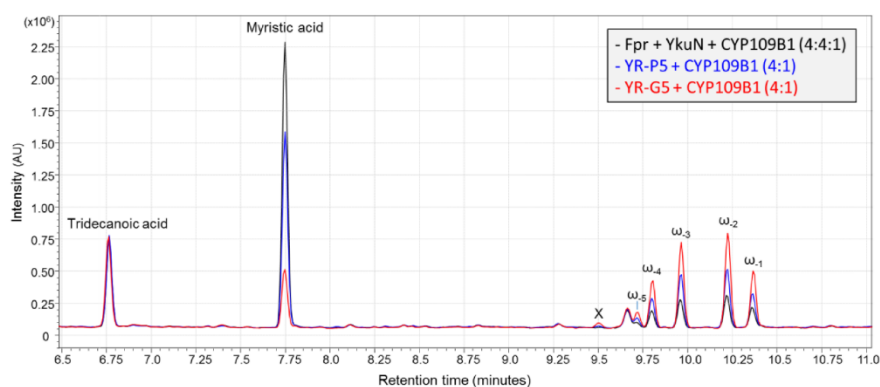


Figure S5. GC-MS analysis of CYP109B1 conversion reactions using different redox partners in the absence of G6P-dehydrogenase. The employed ratio of NADPH:myristic acid was 1:1 (200 μ M each, respectively). After NADPH depletion, the proportion of myristic acid that was converted was determined. Herein, the coupling efficiency equals the proportion (%) of myristic acid converted upon NADPH depletion. Tridecanoic acid (50 μ M) was used as internal standard. Traces are shown for reactions performed with redox partners Fpr/YkuN (black), YR-P5 (blue) and YR-G5 (red), achieving 25%, 47% and 84% conversion, respectively. X indicates a possible overoxidation product of myristic acid, whereas the peak eluting at 9.67 min represents the fatty acid impurity hexadecanoic acid.

REFERENCES

- 1 Sambrook, J. & Russell, D. W. *Molecular cloning: a laboratory manual, 3rd ed.*, (Cold Spring Harbor Laboratory Press, 2001).
- 2 Bakkes, P. J. *et al.* Design and improvement of artificial redox modules by molecular fusion of flavodoxin and flavodoxin reductase from *Escherichia coli*. *Sci. Rep.* **5**, 12158, doi:10.1038/srep12158 (2015).
- 3 Girhard, M., Klaus, T., Khatri, Y., Bernhardt, R. & Urlacher, V. B. Characterization of the versatile monooxygenase CYP109B1 from *Bacillus subtilis*. *Appl. Microbiol. Biotechnol.* **87**, 595-607, doi:10.1007/s00253-010-2472-z (2010).
- 4 Girhard, M. *et al.* Regioselective biooxidation of (+)-valencene by recombinant *E. coli* expressing CYP109B1 from *Bacillus subtilis* in a two-liquid-phase system. *Microb. Cell Fact.* **8**, 36 (2009).
- 5 von Bühler, C., Le-Huu, P. & Urlacher, V. B. Cluster screening: an effective approach for probing the substrate space of uncharacterized cytochrome P450s. *ChemBioChem* **14**, 2189-2198, doi:10.1002/cbic.201300271 (2013).
- 6 Lisurek, M., Kang, M. J., Hartmann, R. W. & Bernhardt, R. Identification of monohydroxy progesterones produced by CYP106A2 using comparative HPLC and electrospray ionisation collision-induced dissociation mass spectrometry. *Biochem. Biophys. Res. Commun.* **319**, 677-682 (2004).
- 7 Brixius-Anderko, S., Schiffer, L., Hannemann, F., Janocha, B. & Bernhardt, R. A CYP21A2 based whole-cell system in *Escherichia coli* for the biotechnological production of premedrol. *Microb. Cell Fact.* **14**, 135, doi:10.1186/s12934-015-0333-2 (2015).
- 8 Sagara, Y. *et al.* Direct expression of adrenodoxin reductase in *Escherichia coli* and the functional characterization. *Biol. Pharm. Bull.* **16**, 627-630 (1993).
- 9 Uhlmann, H., Beckert, V., Schwarz, D. & Bernhardt, R. Expression of bovine adrenodoxin in *E. coli* and site-directed mutagenesis of /2 Fe-2S/ cluster ligands. *Biochem. Biophys. Res. Commun.* **188**, 1131-1138 (1992).
- 10 Zhang, A. *et al.* The crystal structure of the versatile cytochrome P450 enzyme CYP109B1 from *Bacillus subtilis*. *Mol. Biosyst.* **11**, 869-881, doi:10.1039/c4mb00665h (2015).
- 11 Janocha, S., Carius, Y., Hutter, M., Lancaster, C. R. & Bernhardt, R. Crystal Structure of CYP106A2 in Substrate-Free and Substrate-Bound Form. *ChemBioChem* **17**, 852-860, doi:10.1002/cbic.201500524 (2016).
- 12 Haider, S. *et al.* Structure-phenotype correlations of human CYP21A2 mutations in congenital adrenal hyperplasia. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 2605-2610, doi:10.1073/pnas.1221133110 (2013).
- 13 Omura, T. & Sato, R. The Carbon Monoxide-Binding Pigment of Liver Microsomes. I. Evidence for Its Hemoprotein Nature. *J. Biol. Chem.* **239**, 2370-2378 (1964).
- 14 McIver, L. *et al.* Characterisation of flavodoxin NADP⁺ oxidoreductase and flavodoxin; key components of electron transfer in *Escherichia coli*. *Eur. J. Biochem.* **257**, 577-585 (1998).
- 15 Fujii, K. & Huennekens, F. M. Activation of methionine synthetase by a reduced triphosphopyridine nucleotide-dependent flavoprotein system. *J. Biol. Chem.* **249**, 6745-6753 (1974).
- 16 Wang, Z. Q. *et al.* Bacterial flavodoxins support nitric oxide production by *Bacillus subtilis* nitric-oxide synthase. *J. Biol. Chem.* **282**, 2196-2202 (2007).
- 17 Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254 (1976).
- 18 Girhard, M., Tieves, F., Weber, E., Smit, M. S. & Urlacher, V. B. Cytochrome P450 reductase from *Candida apicola*: versatile redox partner for bacterial P450s. *Appl. Microbiol. Biotechnol.* **97**, 1625-1635, doi:10.1007/s00253-012-4026-z (2013).

- 19 Jorgensen, W. L. & Madura, J. D. Quantum and Statistical Studies of Liquids .25. Solvation and Conformation of Methanol in Water. *J. Am. Chem. Soc.* **105**, 1407-1413, doi:DOI 10.1021/ja00344a001 (1983).
- 20 Berendsen, H. J. C., Postma, J. P. M., Vangunsteren, W. F., Dinola, A. & Haak, J. R. Molecular-Dynamics with Coupling to an External Bath. *J. Chem. Phys.* **81**, 3684-3690, doi:Doi 10.1063/1.448118 (1984).
- 21 Parrinello, M. & Rahman, A. Polymorphic Transitions in Single-Crystals - a New Molecular-Dynamics Method. *J. Appl. Phys.* **52**, 7182-7190, doi:Doi 10.1063/1.328693 (1981).
- 22 Hess, B. P-LINCS: A Parallel Linear Constraint Solver for Molecular Simulation. *J. Chem. Theory Comput.* **4**, 116-122, doi:10.1021/ct700200b (2008).
- 23 von Böhler, C. J. & Urlacher, V. B. A novel P450-based biocatalyst for the selective production of chiral 2-alkanols. *Chem. Commun. (Camb.)* **50**, 4089-4091, doi:10.1039/c4cc00647j (2014).
- 24 Lawson, R. J., von Wachenfeldt, C., Haq, I., Perkins, J. & Munro, A. W. Expression and characterization of the two flavodoxin proteins of *Bacillus subtilis*, YkuN and YkuP: biophysical properties and interactions with cytochrome P450 Biol. *Biochemistry* **43**, 12390-12409 (2004).
- 25 Jenkins, C. M. & Waterman, M. R. NADPH-flavodoxin reductase and flavodoxin from *Escherichia coli*: characteristics as a soluble microsomal P450 reductase. *Biochemistry* **37**, 6106-6113 (1998).
- 26 Ingelman, M., Bianchi, V. & Eklund, H. The three-dimensional structure of flavodoxin reductase from *Escherichia coli* at 1.7 Å resolution. *J. Mol. Biol.* **268**, 147-157, doi:10.1006/jmbi.1997.0957 (1997).
- 27 Honda Malca, S., Girhard, M., Schuster, S., Durre, P. & Urlacher, V. B. Expression, purification and characterization of two *Clostridium acetobutylicum* flavodoxins: Potential electron transfer partners for CYP152A2. *Biochim. Biophys. Acta* **1814**, 257-264, doi:10.1016/j.bbapap.2010.06.013 (2011).

3 Discussion and conclusion

The aim of this Thesis was to optimize the electron transfer systems of P450s and it was part of the BMBF-project “SupraRedoxModule”. The project was focussed towards a universal redox module for P450s, as every P450 needs electrons to convert the given substrates. However, because the group of P450s as well as their corresponding redox partners are vast and diverse, an interim goal would be improvements in understanding the redox partner interaction with the P450, as well as facilitating the purification of the redox system and the possibilities for reusing the purified enzymes.

This Thesis contributes to the SupraRedoxModule-project via different subprojects: First, it reports on the redox partner exchanges and redox partner mutations to improve the yield of main product formation in by changing the binding possibilities of the ferredoxin to the reductase and the P450. Second, it shows possibilities of reducing the complexity of the redox chain via different fusion proteins like the DuaLinX-subproject and the reductase-regenerating fusions via two different linker possibilities. Third, the immobilization of the redox enzymes as well as the P450 provides experimental results for recycling involved enzyme components. Taken these results all together, they show ways for a better understanding of P450 systems and, therefore, also improve the possibilities for future biotechnological utilization.

The above mentioned subprojects are partly published (see section 2), partly unpublished. All of them will be discussed in the following discussion.

3.1 Redox partner choices

3.1.1 Modulation of product pattern by the use of different redox partners

The redox systems of different organisms that were already identified as being able to transport electrons to particular P450s were used to identify the best working redox partners for the well-studied CYP106A2. These systems are namely the ferredoxins Adx and Adx(4–108) (both *Bos taurus*), electron transfer protein 1 (Etp1(516–618)) (*S. pombe*), Fdx2 (*B. megaterium*), the flavodoxins FldA (*E. coli*), and YkuN (*B. subtilis*) as well as the reductases AdR (*Bos taurus*), AdR homolog 1 (Arh1) (*S. pombe*), BmCPR (*B. megaterium*), and Fpr (*E. coli*). As postulated, all redox partners could generally support the electron transfer onto the CYP106A2 and the *in vitro* conversion of progesterone, with the truncated ferredoxins supporting almost complete conversion of the substrate (see Figure 5). Additionally, and even more interestingly, the

experiments showed a significant difference in product pattern, in particular a shift from a high amount of undesired polyhydroxylated progesterone products when using Adx(4-108) towards high amounts of the main 15β -hydroxyprogesterone when using Etp(516-618). This (unexpected) result was further investigated by evaluation of time-dependent *in vitro* substrate conversions, which exhibited a rise of polyhydroxylations after an initial moderate to high amount of main product when applying Adx(4-108). In contrast, Etp(516-618) does not lead to further changes in the hydroxylation pattern of the product as times progresses.

Computational protein-protein docking studies were then employed to elucidate the differences observed by the use of different redox partners in the conversion of progesterone by CYP106A2. Despite their amino acid identity of 46 % and highly conserved regions between Adx(4-108) and Etp(516-618), it was proven that Etp(516-618) binds closer to the CYP106A2. This is caused by the presence of F82 in Etp(516-618), resulting in a shorter distance between the FeS cluster of Etp(516-618) and the heme-iron atom of the cytochrome compared with the interaction between Adx and the P450. Both, the closer bond as well as the shorter distance, should lead to a faster electron transfer. This hypothesis was experimentally investigated via the stopped-flow technique, showing that the combination of Etp(516-618)/arh1/CYP106A2 leads to a higher apparent rate constant (k_{app}) than the redox system Adx/AdR/CYP106A2. Another explanation for the changed product pattern could be linked to the fact that the binding of Adx compared to Etp(516-618) seems to be stronger due to larger electrostatic contributions which leads to a slower release of Adx. This last point would let the product remain within the active site of the CYP106A2 longer and, therefore, because of the fast reorientation of the product, produce more hydroxylations.

An overall practical consequence from the above mentioned approach to universal redox systems for P450s is that the choice of redox partners should be thoroughly investigated, both experimentally and by computer modeling, especially regarding time-dependencies, binding behavior, and product patterns of multi-product conversions. To fine-tune the production of pharmaceutically interesting precursors, the redox partner combinations should be chosen to produce high yields of the main product to facilitate further purification and modification steps.

Discussion and conclusion

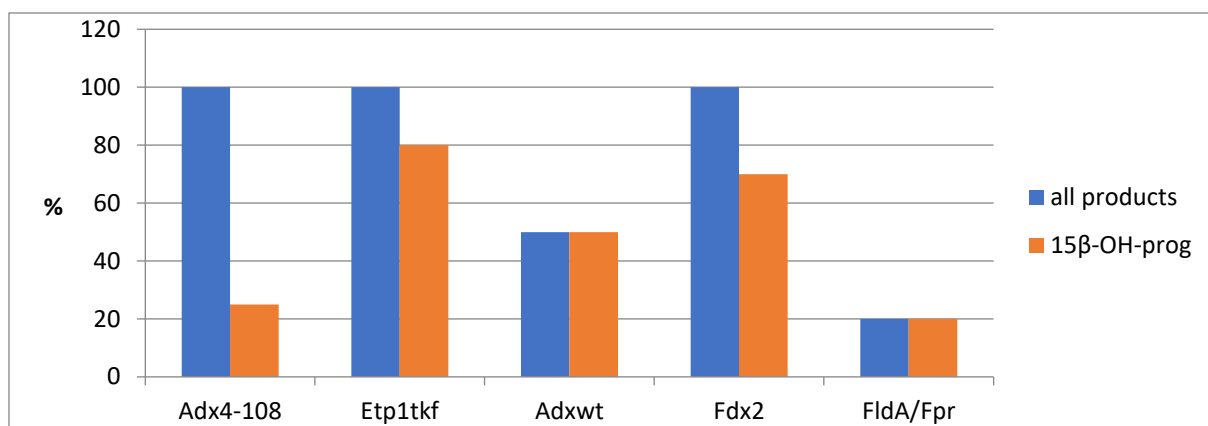


Figure 5: Comparison of overall product yield (blue bars) and main product (15β-OH-prog, orange bars) respective to the used ferredoxin/flavodoxin. The labels of the columns on the x-axis indicate the used ferredoxin (or flavodoxin FldA) in the in-vitro conversion with CYP106A2 and the reductase AdR (or in case of flavodoxin Fpr instead of AdR)

3.1.2 Modulation of product pattern by site-directed mutagenesis of redox partners

It was experimentally demonstrated and further explained by computational methods described in the first publication that the binding modes of the redox partners affect the selectivity of the progesterone hydroxylation by CYP106A2, with Etp1(516-618) leading to higher yields of the main monohydroxylated progesterone product than the usually utilised Adx(4-108) (Sagadin et al., 2018). Based on these findings, the second publication is devoted to the molecular dockings as well as the experiments to optimize the binding of Adx(4-108) by introduction of mutations which successfully lead to a decrease in FeS cluster/heme distance and subsequently also to an increase in the main product yield.

Several ferredoxins share three highly conserved regions that center around the FeS-clusters of the respective proteins, but other regions of the ferredoxins may vary. Comparing the structures and differences of the two concerned ferredoxins, (Etp1(516-618) and Adx(4-108)), their binding towards CYP106A2 is influenced by certain amino acids, which allow or hinder, respectively, the close approach of the FeS-cluster towards the heme. Smaller residues even seem to lower the redox potential. Paying attention to previous results from our lab regarding the amino acid residues Tyr82 of Adx(4-108) (Beckert & Bernhardt, 1997), mutants at this position might induce small conformational changes leading to a different binding of Adx(4-108) to the P450. Moreover, results of the first publication showed that forming hydrophobic contacts of the corresponding residue of Etp1(516-618) enables close contact between the ferredoxin and the CYP106A. The additional mutations of P108 of Adx(4-108) together with the alterations at position 82 should lead to the formation of intermolecular hydrogen bonds or salt bridges. These structures result in a calculated decreased distance of the two cofactors (FeS and heme iron) and are expected to change the selectivity of the progesterone hydroxylation as it is observed using Etp1(516-618).

The five Adx(4108) mutants constructed in the framework of this publication were less stable, which was addressed by a lower expression temperature. Nevertheless, the expected change in selectivity could be proven in *in vitro* assays and showed only a slightly lower main product yield compared to using Etp1(516-618), even exhibiting a similar time dependency as the yeast ferredoxin. The different redox potential between Adx(4-108) and Etp(516-618) was also investigated as a possible reason for the different product patterns, but showed only small or no changes regarding the mutants and the parent Adx(4-108), therefore not serving as a further explanation for these changes (in product pattern). Prediction of the redox potentials of Adx mutants by electrostatic calculations, which was intended to optimize the redox partners, was,

Discussion and conclusion

however, not achieved with the required accuracy. It is difficult to assess whether the available molecular structures generated by molecular dynamics simulations are inadequate for this purpose, or whether the problems arise from the methodology, since no publication where this has been successfully achieved for the adrenodoxin system has been found in the literature.

Furthermore, the protein-protein-dockings confirmed the new formation of intermolecular contacts regarding the altered amino acids at positions 82 and 108, resulting in less steric hindrance when binding between Adx mutants and the P450 occurs. Even the distance of the involved cofactors decreases as predicted. A spacial score obtained from protein-protein-docking of the ferredoxins to the cytochrome also showed a relation with the amount of main product being formed, a fact which emphasizes the correctness of the results.

More mutants of Adx4-108 concerning core amino acid F43 (F43N and F43Q) were proposed, as they were expected to display distinctive opposed effects on redox potentials. It was possible to clone these mutants, but stable expression (and purification) could not be achieved. Therefore, our working hypothesis regarding the change in the local dipole moment in the immediate vicinity of the iron-sulfur cluster and its impact on the redox potential in general and product yield in *in vitro* conversions could not be tested to give more insight into this topic.

The results of these experiments lead to the following conclusions: It is possible to influence the conversion of the substrate by insertion of mutations into the P450-binding area of the ferredoxin part of the redox chain. In this particular case, the mutations for the parent Adx(4-108) were adapted to be similar to the residues of Etp1(516-618), according to computational methods. These newly inserted mutations led to changes in the binding surface, which allowed a closer approach of the cofactor centers, and eventually resulted in the expected change in hydroxylation pattern. This new possibility of designing mutants proves that not only the rational design of P450s is able to alter the product pattern, but that rational design of the involved redox partners' binding area can be equally important. The experiments and their theoretical background shed further light onto the basic understanding of electron transport within this multi-enzyme electron transfer chain of P450s. Overall, the topic can be used to increase the yield and tune the product pattern of biotechnologically interesting products and, therefore, influence the economics of this biotechnological application.

3.1.3 Consequences for the choice of redox partners

In cases where no ferredoxin is involved (class II P450s), it has already been demonstrated by Gricman that potential reductase-interacting residues of the proximal P450-surface can be identified. The structure of these residues results in universal redox partner interaction sites (RPISs) to all P450s (Gricman et al., 2016), which can be used especially to identify non-native redox partners. If ferredoxins are involved (class I P450s), all six RPISs are also present with some minor changes in RPIS4.

The field of redox partner binding has come into focus as it can influence a number of parameters. To transfer electrons to the P450, the redox partners, in many cases the ferredoxin part, have to bind to the P450. For example, it was shown by computational and experimental isothermal titration calorimetry studies that the binding of putidaredoxin (Pdx) takes place on the open P450cam (CYP101) (Hollingsworth et al., 2016; Tripathi et al., 2013). It was even demonstrated that binding of the ferredoxin influences closing and opening of the P450, therefore, playing an essential role and explaining the preference of some P450s to “their” favorable ferredoxins (Myers et al., 2013). Binding at the redox partner’s site leads to conformational changes on the distal site of the P450, resulting in opening of a substrate-entry channel (Pochapsky et al., 2003).

Electrostatic interaction of the redox partners, namely ferredoxin and P450, heavily influences their recognition, where the positive charges on the proximal P450 surface steer the negatively charged FeS-cluster of the ferredoxin into a docking position (Chiliza 2020). The influence of these electrostatic interactions has also been discussed in the first and second paper (see section 2.1 and 2.1). Changes in redox potentials of ferredoxins and P450s can be observed in some instances and might influence the P450 activity: Other examples show that the redox potential is not the key determinant for the P450 activity (Zhang et al., 2018), a fact that we were able to observe as well (second paper, section 2.2). Furthermore, the distance between the iron-sulfur cluster of the ferredoxin as well as the heme-iron of the P450 proved to be very important, as published in this thesis as well as in other publications (Chiliza et al., 2020; Sevrioukova & Poulos, 2011; Zhang et al., 2018). Despite of all these results, the complexity of the ferredoxin-P450 interactions challenges some of the progress already made and necessitates more work to design universal electron transfer partners (Chiliza et al., 2020).

It was recently described that the amount and choice of redox partners, especially for bacterial P450s, may affect catalytic rates, product distribution as well as type and selectivity of P450 reactions. Meaning that the redox partners not only provide electrons but are able to modulate

the P450 function (Khatri et al., 2017; Li et al., 2020). The variety of redox partners that can be present in a bacterial cell might be a reason for the adaptability of the bacteria to difficult or changing environments by enabling detoxification of more diverse xenobiotics or synthesis of more diverse metabolites (Li et al., 2020).

Furthermore, the homologous redox partners are not necessarily the optimal ones for the respective P450, as shown by Zhang et al., revealing that hybrid electron transfer systems are able to outperform the “parent one” (Zhang et al., 2018). They also stated that from a biotechnological point of view the most efficient redox partner might increase overoxidation, which is why choosing a less efficient redox partner can be more preferable (Zhang et al., 2018). In our studies, we were also able to find hints for this theory, as CYP106A2 in combination with Adx(4-108) was more efficient in converting the progesterone compared with Adx (wildtype). In contrast, there was a lot of product with several unselective oxidations (i.e. “overoxidation”) when using the “fast” Adx(4-108), in comparison to wildtype Adx, the less efficient ferredoxin in this case (see Section 2.1).

Overall, redox partner choices can be very critical for the outcome of P450 conversion. Both homologous redox partners and heterologous choices should be examined thoroughly as well as aspects like RPISs to select those most suitable for the desired experiment or biotechnological approach.

3.2 Fusion of redox partners

3.2.1 Fusion of redox partners using DuaLinX

Fusion or any kind of assembly of the electron transfer chain of P450s can be performed in different settings, meaning that for the three components of the class I system, multiple combinations can be fused or assembled artificially to enhance the activity of the system and reduce the complexity of multi-component structure. P450 BM3 serves as good model for fusions as is a fusion protein (of a P450 and a reductase domain) itself, has a high catalytic efficiency and is very good characterized structurally (J. S. Miles et al., 1992), therefore serving as a blueprint to fuse other P450s with respective redox partners. Several approaches have already been developed to facilitate artificial fusions of P450 electron transfer chains, out of which popular models are Molecular Lego, PUPPET, and LICRED. Molecular Lego is a system which generates redox transfer systems by shuffling well-characterized building blocks of redox partners for applications in bioelectrochemistry (Gilardi et al., 2002; Sadeghi et al., 2000).

PUPPET, on the other hand, uses three distinct proliferating cell nuclear antigens from *Sulfolobus solfataricus* that can form a heterotrimer while each unit is fused to one protein (the P450 or one of two redox proteins), resulting in a better electron transfer via the complex itself (Hirakawa et al., 2013; Hirakawa & Nagamune, 2013). LICRED describes a system, where a ligation-independent cloning vector is used to develop self-sufficient P450-reductase libraries for the high-throughput screening of uncharacterized P450-domains (Sabbadin et al., 2010).

Another approach is the introduction of various sets of linkers. Linker peptides are normally introduced into fusion proteins to avoid steric hindrance between the domains and then may use the natural multi-domain proteins as a guide, but can also use artificial flexible or rigid sequences (Chen et al., 2013). Before the YkuN-Fpr fusion proteins were constructed as described in Section 2.3, the “DuaLinX” tool was developed by the Urlacher group to study the effect of linker length, type, as well as order of the *E. coli* redox partners Fpr and FldA (Bakkes et al., 2015). By virtue of this tool, the sets of fusion proteins were constructed either having glycine or proline rich linkers of different lengths (5, 10, 15, 20, 25 amino acids). Results showed that a specific order (FldA:Fpr) performed better than *vice versa* (reasons might be the C-terminal cofactor-binding residues being affected by the linker) and that longer linkers outperformed shorter ones. The electron transfer seems to rely on a certain distance between the redox centers (Moser et al., 1992), and the linkers of longer length seem to provide this spatial arrangement better. Furthermore, the proline-rich linkers might more effectively isolate the involved protein domains (Tanaka et al., 2006), and, therefore, interfering less with protein function than flexible glycine-rich linkers.

Published and described in Section 2.3 (Bakkes et al. 2017), the formerly described DuaLinX tool was used to modify the first attempt of a non-physiological electron transfer system. FldA was changed to FldA to the YkuN, a flavodoxin from *B. subtilis*, as it often outperforms FldA/Fpr (von Bühler et al., 2013). One reason for the preference of YkuN over FldA in conversion with CYP109B1 could be that both originate from *B. subtilis*. Similar to the first approach with FldA and Fpr, the fusion protein of YkuN and Fpr was constructed in a way that the flavodoxin was fused to the N-terminus of Fpr, hereafter abbreviated further as YR (YkuN:Fpr). To elucidate which kind of linker type and length exhibits the best catalytic performance, several P450s were chosen to convert substrates using the fusion protein. Computational methods were applied as well. As an excess of ferredoxin is usually required to sufficiently drive the conversion of the substrate by the P450s and the stoichiometry of the fused redox partners is fixed at 1:1, the required amount of unfused redox partner was first examined

in *in vitro* conversions and found to be almost equal, using 4:4:1 (Fpr/YkuN/P450) compared to the usual ratio of 1:10:1. Therefore, the fusion constructs with linker length and type variety were tested with the ratio of 4:1 (YR/P450). The first P450 to be tested was the CYP109B1 with myristic acid as substrate, where it was shown that all fusion constructs could support the electron transfer towards the P450. Similar to the fusion with FldA, the linker-less fusion with YkuN did not reach the same conversion rate as the non-fused partners, but all linker-fused proteins could perform better than the linker-less one. Especially the long and rigid fusions YR-P4 and YR-P5 supported the conversion of the model substrate similarly to the non-fused Fpr/YkuN, a fact which was analogously observed with the fusion FldA:Fpr (Bakkes et al., 2015). The superiority of the proline-rich linkers over the glycine-rich linkers was verified by molecular dynamics simulations. Glycine-rich linkers suffered a collapse of the linker structure paired with decreasing radii of gyration and oscillations over time. These structural parameters exhibit high structural rearrangements and flexibility, which potentially lead the fused domains to sterically crash more easily into each other and, therefore, impair the electron transfer and by extension the conversion of the substrate. Proline-rich linkers, on the other side, proved to remain extended with only minor oscillations, thus attributing to the better performance of them.

In most reconstituted P450-systems, the availability of the electron shuttle protein (ferredoxin or flavodoxin) limits the conversion, a fact that could be alleviated by an excess of this protein (Jenkins & Waterman, 1994). By either applying extra YkuN or increasing the concentration of the fusion protein, the limitation was able to be overcome. This also adds proof that Fpr in the fusion protein is not only able to donate electrons to the fused YkuN, but also to additional free YkuN, if this is present in the mix. The coupling efficiency and NADPH oxidation rate was determined, showing that the P450 with the unfused redox partners exhibits the highest NADPH oxidation rate and a coupling efficiency of approximately 28 %. Fusion constructs lowered the NADPH oxidation rate while improving the coupling efficiency. To pursue the goal of a universal redox system for P450s, additional P450s were tested as terminal electron acceptors, such as CYP154E1 from *Thermobifida fusca* YX, CYP106A2 from *B. megaterium*, and bovine CYP21A2. Fpr and YkuN as free enzymes are already known to work with CYP154E1, and moreover that their fused domains proved to support the conversion of the model substrate β -ionone with similar parameters as the non-fused redox partners. This approach confirmed the results that the YR-P5 construct outperformed the conversion of the other tested fusion constructs, similarly to the initial conversion of myristic acid with CYP109B1.

The YR-P5 fusion protein with the highest apparent conversion rates was further shown to support the conversion of progesterone by CYP106A2, substituting the regularly used AdR/Adx redox partners (parent system). It is worth noting that non-fused YkuN/Fpr supports an almost identical conversion of progesterone by CYP106A2 (compared to the parent system) regarding both overall conversion rate as well as product distribution. The fusion protein YR-P5 cuts the conversion rate in half using a ratio of 4:1 YR-P5:CYP106A2, in contrast to using the AdR/Adx system, producing almost exclusively main product. Increasing the ratio to 10:1, the conversion rate was similar to the parent one with small amounts of polyhydroxylated product. The membrane-bound CYP21A2, where the parent redox system is the microsomal CPR, but which can also use AdR/Adx, was also able to be supported by the YR-P5 fusion, achieving about 40 % conversion of the substrate progesterone.

Therefore, it is appropriate to state that the YR-P5 fusion supports the catalytic activity of CYP154E1, CYP106A2, and CYP21A2, in the latter two cases substituting the mammalian electron transfer systems of AdR/Adx and CPR. Nevertheless, the non-fused proteins were more effective in contributing to the conversions than the fused ones, presumably because of their faster electron transfer, a fact which also supports the formation of polyhydroxylated progesterone products formed by CYP106A2 with non-fused YkuN/Fpr compared to the fusion protein.

3.2.2 Artificial and natural fusions of P450s with their redox partners

As the native redox partners of P450s are rarely obtained, surrogate redox partners are widely used for P450s, always assuming that their choice does not inflict the type and selectivity of the P450's reactions. On the other hand, it was proven that fusions of redox partners to P450s lead to a limitation of the mediated catalyzations. The protein-protein interaction could, therefore, serve as a modulator of the catalytic function of P450s by indirectly modifying the active site (Zhang et al., 2014). A probable explanation would be that several redox partners are available in an given organism. This point give the chance to alter the electron transfer as well as the binding of redox partners and P450 in different ways, thereby serving as a possibility to accept diverse substrates to adapt to a changing environment. In contract, fused redox partners only lead to conversion of a limited variety of substrates (S. Li et al., 2020; Zhang et al., 2014).

Nevertheless, P450-redox partner fusion enzymes also occur naturally (Jackson et al., 2002; Rylott et al., 2006) with the flavocytochrome P450 BM3 (CYP102A1) as the first to be discovered (J. S. Miles et al., 1992; Ruettinger & Fulco, 1981). Since the ferredoxin is postulated to generally shuttle electrons from the reductase to the P450, the discovery of a fusion protein of a P450 domain to a ferredoxin was quite surprising, describing a completely new class of P450s (Jackson et al., 2002).

The biotechnological advantages of artificial fusion enzymes lie in simplifying the overall enzyme system and the expression. However, they are not yet used extensively (Munro et al., 2007), as they do not yet reliably achieve the catalytic activity of the free enzymes in most cases (McLean et al., 2007). In fusion enzymes, activity is also shown to be dependent on the used linkers, i.e. their rigidity, composition, or length (McLean et al., 2007). Early attempts were already made to fuse the eukaryotic CYP11B1, Adx, and AdR, using various linkers and orders (Cao et al., 2000), resulting in active proteins with lower conversion rates compared with the free enzymes. In further research works, bacterial P450s were fused with their surrogate redox partners in order to facilitate their use in biotechnological approaches, e.g. CYP101 with Pdx and PdR. The latter one resulted in active proteins that were even more efficient in some concentrations than the reconstituted system (Sibbesen et al., 1996).

The approach described of using glycine-rich flexible or proline-rich rigid linkers (see section 2.3) was successfully used in further experiments by Kokorin to fuse BM3 to the formate dehydrogenase, resulting in a self-sufficient biocatalyst displaying an overall higher activity than the separate enzymes (Kokorin et al., 2021).

In summary, the natural as well as the artificial fusion enzymes are very complex, but efficient redox partner chains are possible by the modulation of the involved enzyme components. Therefore, aiming for the final goal of biotechnological use of P450 systems is reasonable.

3.2.3 Creation of a chimera between AdR and alcohol dehydrogenase of *Lactobacillus brevis*

Another challenging difficulty with P450s is the availability of the cofactor NADPH (*in vivo* as well as *in vitro*) that can be a limiting factor in their biotechnological application. Both the cost and the availability of the NADPH in the cell play a major role. As previously proven in our lab, co-expression of CYP105A1 not only with its redox partners Adx and AdR, but also with alcohol dehydrogenase from *Lactobacillus brevis* (*LbADH*), in order to gain NADPH recycling possibilities for the expensive redox equivalent, resulted in substrate conversions with high product yields (Janocha & Bernhardt, 2013). *LbADH* is particularly characterized by its stability under a wide variety of conditions, e.g., even in unconventional media and organic solvents (Leuchs & Greiner, 2011). This facilitates a NADPH regenerating system that can be used for the maintenance of the electron transport chain through NADPH regeneration in biotechnologically used cytochrome P450 systems. Therefore, a further approach was endeavoured during this thesis for an improved and simplified redox chain regarding the SupraRedoxModul(e) to test whether a fusion protein from AdR and *LbADH* could further increase these yields. In addition to the fusion protein would be of particular interest for immobilization, since it can be used to fix a regeneration system directly with the reductase component.

This regeneration system keeps the cofactor NADPH in a so-called "cage" and could be used in *in vitro* conversions with P450 enzymes to minimize the need for the expensive cofactor. It could also secure the NADPH supply *in vivo*, uncoupling it from other metabolic pathways and leading to more efficient substrate conversion due to the shorter paths of NADPH between the AdR and the *LbADH*. It has to be taken into account that an experimental approach should address different complications: mobility of the involved enzyme to ensure their own catalytic properties, flexibility for the shuttle of NADPH between AdR and *LbADH*, and avoidance of steric hindrance from different protein regions of the other protein. To link the two distinctive domains, rational design of rigid and flexible linkers was performed. Flexible linkers would allow interaction between domains or offer spatial separation of domains. In contrast, rigid linkers would maintain the distance between the involved domains and prevent inefficient

separation (Chen et al., 2013). Both properties can be favorable and have to be considered when choosing the linker for distinct domains. In the end, the linker (GGG(PPPPS)3GGG) used for this project was designed to allow interaction between the reductase and the alcohol dehydrogenase via flexible glycine "joints" (made of 3 glycine residues), which facilitate the transition between the proteins and the linker, and the rigid inner part of the linker (P_n) that maintains the distance so that the two domains would not clash or interfere too severely (see Figure 6). The so-called proline linker was cloned between the C-terminus of the AdR and the N-terminus of the *Lb*ADH.

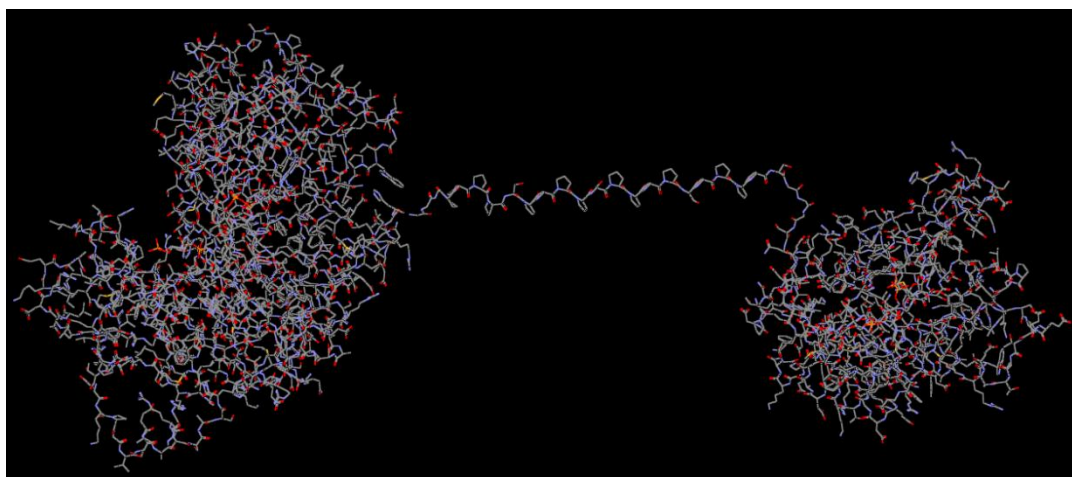


Figure 6: AdR-proline linker-LbADH fusion protein model (screenshot). On the left is AdR (461 amino acids), in the middle is the proline linker (23 amino acids), and on the right is LbADH (252 amino acids).

As initial expression attempts showed additional bands in the Western blot with an AdR antibody, incomplete fusion protein variants were assumed, particularly containing AdR fragments. One of the reasons for the merely partial formation of the fusion protein could be the use of a single codon for proline in the linker region. It is possible that there is not enough tRNA to link proline to proline in the growing amino acid chain in rapid succession. This problem was overcome by employing three codons instead of one codon for proline. In addition, a 6xHis tag N- or C-terminal using PCR was added to facilitate purification. The best expression, independent of the N- or C-terminal 6xHis tag, was achieved after 48 hours at 26 °C. The coexpression of the chaperone system GroEL/ES showed only minor changes in the expression level. In a spectrometric comparison with pure AdR, partially matching maxima could be confirmed. However, examination by SDS-PAGE exhibited many additional bands, some probably being the result of incompletely formed fusion proteins. Because of these many impurities and only unsuccessful attempts for optimizations, *in vitro* conversions were not conducted as they were not considered to yield promising results.

In the end, this project of a fusion protein produced from AdR and *Lb*ADH to regenerate the NADPH cofactor proved to be not successful despite numerous linker optimizations and, therefore, was discontinued.

3.2.4 Creation of a chimera between Arh1 and alcohol dehydrogenase of *Lactobacillus brevis* (Bachelor's thesis)

Since the expression of the fusion gene of bovine AdR and alcohol dehydrogenase from *Lactobacillus brevis* was not stable, another approach to a fusion protein for the regeneration of NADPH was created. Using the DuaLinX system, fusion genes of the NADPH-consuming AdR-homologue Arh1 from *S. pombe* and ADH from *Lactobacillus brevis* were produced with both glycine- and proline-rich linkers of different lengths. Arh1 was chosen due to the fact that expression and purification is simpler compared with AdR and, therefore, more promising than in the other approach. The DuaLinX system already proved to provide a good approach to fuse proteins and was already applied to two sets of redox partners. Generally, similar to the former approach with AdR and *Lb*ADH, a spatial proximity between these two proteins was to be established and a so-called "caging" of the NADPH be facilitated. In order to investigate the influence of the reductase and the dehydrogenase's position, the C- and N-terminal positions were swapped (Arh1-Linker-*Lb*ADH (RD) and *Lb*ADH-Linker-Arh1 (DR)). A total of 14 fusion constructs out of 20 possible ones were obtained during a bachelor thesis.

The fusion proteins were expressed to validate the functionality, and an expression control was carried out using SDS-PAGE gel electrophoresis. In order to test the functionality of the constructs, they were integrated into a functional system consisting of the construct, the electron transfer protein Etp1, and CYP106A2. To check the functionality of the Arh1/ *Lb*ADH fusions, samples of defined cell numbers were lysed and the lysate obtained was used in an *in vitro* assay with CYP106A2. The *in vitro* assays were designed to test two functions. First, the function of the reductase domain was investigated in one series of conversions in the presence of G6PDH and G6P, able to regenerate NADPH. Second, the desired function of NADPH regeneration between the reductase and dehydrogenase domains was evaluated in another series (without the NADPH regeneration system). Unfortunately, the expression control by SDS-PAGE gel electrophoresis could not prove any clear expression of the fusion constructs, and the *in vitro* function control assays also showed no activity of the potential fusion proteins.

There are several possible reasons for these results: First, the proteins Arh1 and *Lb*ADH could hinder each other sterically due to the spatial proximity that results from the fusion, thereby rendering NADP⁺ binding to *Lb*ADH impossible. The switch of both enzyme domains (Arh1-

Linker-*Lb*ADH (RD) and *Lb*ADH-Linker-Arh1 (DR)) did not show preferences for one order, leading to the assumption that the fusion of *Lb*ADH in both orders hinders the NADP⁺-binding. Second, even the samples with G6PDH (which should demonstrate the functionality of Arh1) did not exhibit any conversion, leading to the conclusion that Arh1 is unable to function properly. A third reason for this negative outcome might be the close combination of two proteins that are no natural interaction partners. Even in non-fused *in vivo* approaches using the *Lb*ADH as NADPH-regenerating system, the two proteins are expressed within one cell, but probably are not directly in contact with each other compared to both of these fusion approaches.

Nevertheless, a change in the expression conditions of the potential fusion proteins could lead to better outcomes. As the SDS-PAGE only showed an extremely weak band of the fusion proteins, suggesting that the proteins are only expressed at very low levels, a stronger expression promoter might achieve an expression-enhancing effect. Also, the use of another expression cell line in order to increase the yield of fusion protein could attribute for this effect.

Additional starting points for pursuing this approach could be based on a characterisation of the fusion protein after its purification using a histidine tag. This histidine tag facilitates the purification of the potential fusion proteins, enables the following characterizations and *in vitro* conversion studies, and eliminates the possible disturbances that might occur in the lysate assays. A further bioinformatic approach about the spatial configuration of the fusion proteins has the potential to optimize their interaction.

3.3 Use of immobilization as reusable enzyme source in general

In an additional unpublished project in the SupraRedoxModul, we determined the possibilities to immobilize P450 (and its redox partners) in order to stabilize the enzyme and make it available for several conversions, in other words, fit for a reusable approach.

One challenge of the immobilization of enzymes is the coordination of the binding, which should be quite strong to prevent leaching of the enzyme, but also weak enough to allow the required flexibility of the enzyme for its catalytic activity. The activity of immobilized enzymes depends on different factors: the protein to be immobilized, the immobilization material, the substrate used for the conversion, and the procedure of the immobilization. The size, shape, and structure of the chosen enzyme for immobilization will influence the selection of the support material, especially the pore size and the functional groups (Ispas et al., 2009). Using an

optimized immobilization protocol, the operational and storage lifetime as well as the heat stability of the immobilized enzymes can be highly improved (Ispas et al., 2009). This fact presents a big advantage of immobilized enzymes compared to free enzymes, along with the easy separation possibility from the reaction mixture, which is basically impossible for free enzymes (Bornscheuer, 2003). Furthermore, a one-step extraction and immobilization procedure was described using His-tagged enzymes, facilitating the process towards the fast creation of reusable enzyme complexes even more (Cassimjee et al., 2011). Co-immobilizations of two or more enzymes may enhance the overall performance of enzyme cascades and can be performed simultaneously, in separate immobilizations (for subsequent mixing), and as well as in sequential immobilizations. Each procedure has to be adapted to the enzyme combination used, as the type of immobilization, the ratio of the two enzymes to be immobilized, and the degree of loading of the carrier are decisive for the activity of the enzyme conjugates (Betancor & Luckarift, 2010; Kazenwadel et al., 2015).

Potential disadvantages of enzyme immobilizations are: The loss of enzyme, because the immobilized protein might leach from the support, the conceivable occurrence of diffusion barriers for substrate or product distribution, as well as potentially arising restrictions because of pore dimensions might appear (Ispas et al., 2009). An additional point to consider is that the substrate or product of the conversion must not be absorbed by the immobilization material (Maurer et al., 2003).

Several challenges have been met and adapted to: There has to be enough room for substrate access as low substrate diffusion will reduce activity of immobilized enzyme (Hudson et al., 2008). The leaching might be addressed by the use of mesostructured cellular foam (MCF) material which has smaller pore entrances and, therefore, shows improved overall catalytic activity by reducing leaching. Tunable pore sizes have been developed with 3D interconnected pore structure and cage-like connecting windows, which enabled pressure-driven methods for immobilization (Y. Han et al., 2006), gaining the advantage of increased enzyme loading and reduced leaching in contrast to the conventional stirring method. Flux imbalances should be considered, as they inflict heterologous metabolic pathways, which are partly no subject to the natural regulatory mechanisms (Dueber et al., 2009). A possibility to regulate the metabolism of such a pathway could be the spatial subdivision of the involved enzymes by the use of a protein-scaffold, so that the proteins are colocalized, increasing the electron fluxes (Dueber et al., 2009). Potential applications for any kind of immobilized enzymes are e.g., analytics like

biosensors, bioassays, or bioreactors. As the process is adaptable and mostly scalable, microtiter plates can also be used (Brandt et al., 2006).

Immobilization of P450s has already been proven in various settings. E.g., CYP2C9 and CYP2B4 have been immobilized on a mesoporous material (MCM-41) via the Lewis acids on the material. The catalytic activity was restored without addition of a CPR showing that the material itself probably served as electron transfer partner between NADPH and the enzyme (Rosales Hernández et al., 2005). More recently, P450 BM3 along with glucose dehydrogenase (GDH) from *Thermoplasma acidophilum* has been co-immobilized on various functionalized agaroses to serve as a self-sufficient biocatalyst, showing retained activities up to 100 % and reusability for at least four additional conversions, proving the operational stability of the immobilized enzymes (Solé et al., 2019). Microgels have also been used to immobilize the P450 BM3, resulting in full activity after immobilization and reusability at least seven times, with the 7th reuse cycle still exhibiting about 50 % conversion of the initial activity (Nöth et al., 2020). Different set-ups and application possibilities of P450 immobilization strategies come with challenges, but also with benefits (Valikhani et al., 2021). Two distinct fields are determined for application of immobilized P450s: First, P450-based biosensors which employ human or mammalian P450s to be used in clinical studies to analyse drug metabolism, also including the organ-on-a-chip technology. The second area is the P450-based catalysts which use the attractive bacterial P450s, known for their solubility, high turnover, and good expression in *E. coli* (Valikhani et al., 2021).

As P450s and their electron transfer proteins as well as possible NADPH-recycling enzymes are numerous, there is no universal immobilization strategy that fits all. The immobilization and following activity of any enzyme, in particular of P450s, is influenced by several factors, such as the initial enzyme concentration, immobilization process (pressure-driven, stirring), and the pH value of the used enzyme solution. In collaboration with bioinformatics, the material and the enzyme can be modelled *in silico* to predict the needed pore diameters, accessibilities for enzymes and substrates, and orientation of the enzyme on the support (Weber et al., 2010).

3.3.1 Immobilization of CYP106A2 via Strep-Tag to MCF-material

In the “SupraRedoxModul” BMBF-project, which this thesis was part of, immobilizations of P450s were performed. In a second step, the immobilization of the respective redox partners or their fusions were planned. The MCF-material was modified with cobalt(II)ions to enable immobilization of His-tagged proteins and with streptavidin (STA) for Strep-tagged proteins.

These modifications of the material should allow the chemisorption of the enzymes and prevent the immobilized enzymes to leach from the material. The MCF material has large pore volumes, large surface areas, and large pore sizes, and therefore, is particularly well-suited for the immobilization of enzymes (Y.-J. Han et al., 2002). Successful immobilization of various enzymes like lysozyme (Kim et al., 2011), lipase (Kannan et al., 2014), glucose oxidase (Jung et al., 2009), and α -amylase (Pandya et al., 2005) were described in the literature. Additionally, cooperative tandem catalysis with a co-immobilized enzyme and a metal where the latter one mimics a metalloenzyme (Engström et al., 2013) was described.

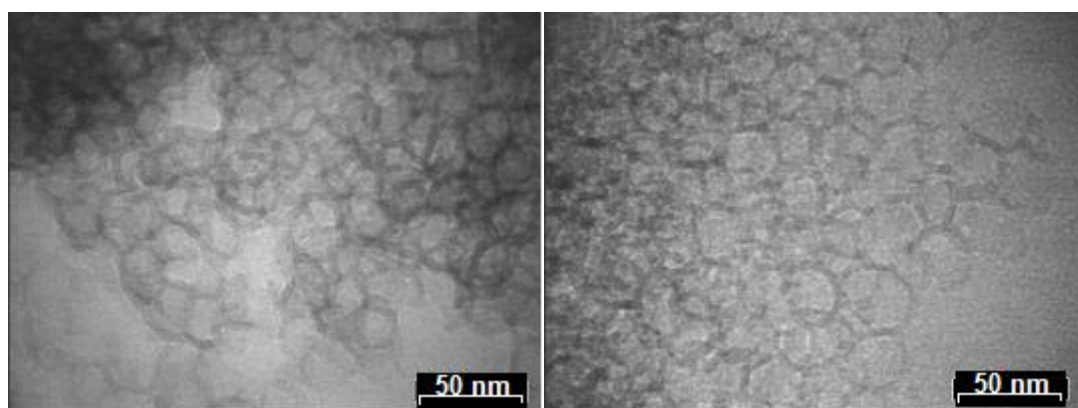


Figure 7: TEM images of MCF 13 with 20 nm cells (left) and MCF 16 with 30 nm cells (right). The scale bars are 50 nm. The measured cell sizes are in line with the ones calculated in preparation. MCF materials are used during the experiments for the surface modification with streptavidin and cobalt(II)ions in order to immobilize the CYP106A2 and Arh1, respectively. The TEM images were prepared by SupraRedoxModul-project partner Kerstin Thiele in Leipzig.

In our immobilization approach and in order to show the suitability of large pore spherical MCF materials (see Figure 7) for enzyme immobilization, the surface was modified, on the one hand, with streptavidin (STA), and on the other hand with cobalt(II)ions. The STA-modified support is able to create a binding towards a Strep-tagged enzyme and the grafted cobalt(II)ions can immobilize enzymes with a His-tag by coordinative bonding. The well-characterized P450 CYP106A2 carrying a N-terminal Strep-tag was used in concentrations of 20 μ M, which is within the range of usual *in vitro* conversion assays. The best immobilization procedure was the incubation at 10 °C for 20 hours, whilst rotating at 4 rpm in an overhead shaker. After the centrifugation, the supernatant was carefully removed and the obtained MCF-materials with immobilized enzyme were washed with a buffer solution. The supernatant and the washing solution were combined and used to determine the concentration of non-immobilized enzyme. The amounts of immobilized enzyme were obtained by subtracting the amount of non-immobilized enzymes from the total amount of enzyme added during immobilization.

The ability of MCF-25STA to bind Strep-tagged proteins was demonstrated by the immobilization of strep-tagged CYP106A2. In addition, this enzyme was immobilized on silica gel 62 serving as a reference silica material. Under the tested conditions the MCF-25STA bound all of the applied cytochrome P450, whereas binding to silica gel was less efficient. After immobilization, the supports were assayed for CYP106A2 activity using progesterone as a substrate as well as the required redox partners Adx4-108 and AdR. For this purpose, a fixed amount of immobilized CYP106A2 was applied for each reaction and the activity was determined after 10 min (Figure 8). The comparison of the activity of the free and the immobilized enzyme exhibited a decrease of the conversion of progesterone, but also a change in the selectivity. Thus, the free enzyme predominantly catalyzes the formation of the unwanted polyhydroxylated progesterone (65 %) and only 28 % of the desired 15 β -hydroxyprogesterone. However, after immobilization of CYP106A2, no polyhydroxylated progesterone was formed and 15 β -hydroxyprogesterone was obtained with 68 % selectivity (Figure 8).

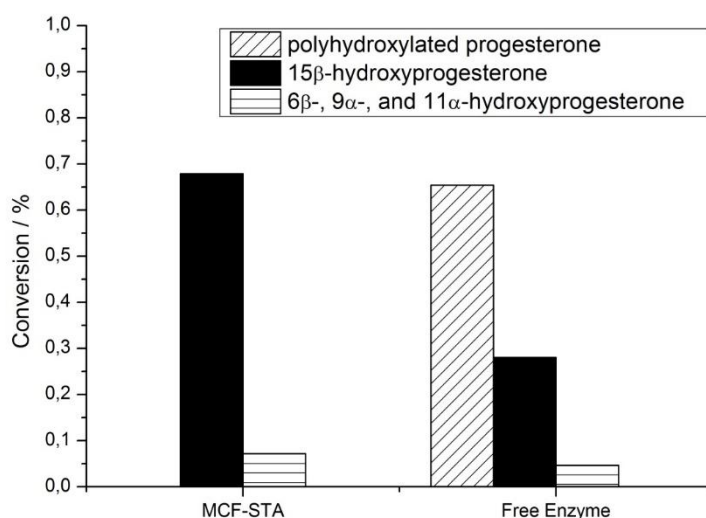


Figure 8: Progesterone conversion (%) by free CYP106A2 in solution or immobilized on MCF-25COO-Co after 10 minutes of reaction time. Black columns represent 15 β -hydroxyprogesterone, diagonally striped columns polyhydroxylated progesterone, and the horizontally striped columns the combined 6 β -, 9 α -, and 11 α -hydroxyprogesterone. Adx4-108 and AdR, both in solution, were added to complete the electron transfer chain. For the sufficient supply of electrons, a glucose-6-phosphate dehydrogenase-based NADPH-regenerating system was used.

The immobilized Strep-tagged enzyme CYP106A2 exhibited less activity than the free enzyme but led to a change of the selectivity in favor of the desired product. This study demonstrated that adjusted modification of spherical MCF particles was suitable for site-specific enzyme immobilization.

Combining the two approaches of His- and Strep-tagged enzymes, one could think of a co-immobilization of a His-tagged CPR-like reductase/ferredoxin part and a Strep-tagged P450, which would create a complete redox transfer chain to convert substrates *in vitro*. The support material MCF would also qualify to be used in a column, so that the ultimate goal would be a flow-through-conversion: immobilizing the P450 with the redox partners (e.g., fusion of ferredoxin and reductase) to a support, filling this support with the immobilized enzymes into a column (like the HPLC-columns or the enzyme purification columns), passing a substrate solution through the column, and “harvesting” the product out of the column’s flow-through. Because the P450 and its redox partners are reusable, a certain amount of purified enzyme should convert much more substrate than the usual *in vitro* conversions. Approaches like this could lead to a more sustainable production of fine chemicals or pharmaceutical precursors.

First attempts for the dual use of Strep- and His-tags were performed by the co-immobilization of the reductase Arh1 on a modified carrier via His-tag in addition to the enzyme Strep-tagged CYP106A2. Here, an activity (although lower than that of the free enzyme) and a further increase in selectivity could also be detected. Various concentration ratios between 10/20 and 20/10 were chosen for the co-immobilization in order to investigate the dependence of the catalytic activity on this ratio. As an activity test, the conversion of the steroid progesterone was investigated. Activity and selectivity can be increased by adjusting the concentration ratio between CYP106A2 and Arh1 during immobilization. Investigations into the reusability of the immobilized enzymes produced only a slight drop in activity of 5.4% after the second use and a further 12.5% after the third use, while maintaining the selectivity.

This work regarding the immobilization via Strep-tag as well as His-tags is not yet published despite its potential to facilitate the *in vitro* conversion possibilities regarding the P450s and its redox partners.

3.4 Conclusion

In summary, the work of this thesis provides insights into the usability and binding specificities of the electron transfer chain involved in conversions with P450s, the possibilities of fusion enzymes regarding the electron transfer chain of P450s as well as the potential of immobilized enzymes in the P450 system.

It was demonstrated with abundant clarity that the often-assumed interchangeability of redox partners providing the required electrons to P450s should not be taken for granted and that this might be the fulcrum of unsuccessful or insufficient substrate conversion. To forego the time-consuming trial-and-error *in vitro* conversion, bioinformatic approaches can be employed to predict the most suitable redox partner for a specific P450. Furthermore, the redox partners, especially the ferredoxin, proved adaptable via site-directed mutagenesis to fit to the precise need of the P450 and the intended conversion. We were also successful in proving that this site-directed, bioinformatics-enabled mutagenesis of Adx(4-108), led to the selective conversion of progesterone by CYP106A2 towards a high yield of the main product 15 β -hydroxyprogesterone. This approach can be utilised to fine-tune further multi-product P450-conversions.

The second major topic of this thesis was the partially successful attempt to fuse parts of the electron transfer system of P450 in order to facilitate the purification. The fusion of YkuN to Fpr with the DuaLinX systems was an improvement over the formerly fused Fdx/Fpr system and proved to be a successful electron transfer partner to several P450s of different origins. Additional fusion projects involved the combination of an alcohol dehydrogenase with the reductase to overcome the need to regenerate the expensive NADPH. Attempts were made using AdR and *Lb*ADH with a proline-glycine linker as well as Arh1 and *Lb*ADH with the DuaLinX system. Both fusion projects did not succeed for of various reasons, illustrating and emphasizing the complexity of the overall project of NADPH-regeneration. Generally, fusions of electron transfer partners definitely should be considered, but bioinformatic tools should be used even more to remove obstacles and increase the success rate of the project.

The last major topic addressed the immobilization of enzymes, particularly of the P450, with this thesis focussing on the CYP106A2. After some adjustments to the immobilization procedure, the CYP106A2 was immobilized successfully via the Strep-tag and capable of converting the model substrate progesterone. The immobilization material MCF was subsequently modified for use in a co-immobilization approach, where CYP106A2 via Strep-

tag as well as the reductase Arh1 via His-tag was utilized successfully. Immobilizations of P450 enzymes and their redox partners on MCF materials show great potential for applications in biotechnological approaches as they exhibit good reusability along with further possibilities to be used in columns. Therefore, they represent a sustainable way to tackle the difficulties of *in vitro* conversion of P450s.

In conclusion, several key points have been addressed in this thesis to support the overall goal of using P450s in biotechnology: Focussing primarily on the involved redox partners, providing particular insights into fusions of them, and shedding light on their immobilizations.

4 References

- Anzenbacher, P., & Anzenbacherová, E. (2001). Review Cytochromes P450 and metabolism of xenobiotics. *Cellular and Molecular Life Sciences*, 58(5), 737–747.
- Ba, L., Li, P., Zhang, H., Duan, Y., & Lin, Z. (2013). Semi-Rational Engineering of Cytochrome P450sca-2 in a Hybrid System for Enhanced Catalytic Activity: Insights Into the Important Role of Electron Transfer. *Biotechnology and Bioengineering*, 110(11), 2815–2825.
- Bakkes, P. J., Biemann, S., Bokel, A., Eickholt, M., Girhard, M., & Urlacher, V. B. (2015). Design and improvement of artificial redox modules by molecular fusion of flavodoxin and flavodoxin reductase from *Escherichia coli*. *Scientific Reports*, 5(1), 1–13.
- Beckert, V., & Bernhardt, R. (1997). Specific Aspects of Electron Transfer from Adrenodoxin to Cytochromes P450scc and P45011. *The Journal of Biological Chemistry*, 272(8), 4883–4888.
- Berg, A. (1982). Characterization of the ferredoxin component of the steroid 15 β -hydroxylase system from *Bacillus megaterium*. *Biochemical and Biophysical Research Communications*, 105(1), 303–311.
- Berg, A., Carlström, K., Gustafsson, J.-A., & Ingelman-Sundberg, M. (1975). Demonstration of a Cytochrome P-450-dependent Steroid 15 β -Hydroxylase in *Bacillus megaterium*. *Biochemical and Biophysical Research Communications*, 66(4), 1414–1423.
- Berg, A., Gustafsson, J.-A., & Ingelman-Sundberg, M. (1976). Characterization of a Cytochrome P-450-dependent Steroid Hydroxylase System Present in *Bacillus megaterium*. *The Journal of Biological Chemistry*, 251(9), 2831–2838.
- Berg, A., & Ingelman-Sundberg, M. (1979). Purification and Characterization of Cytochrome. *The Journal of Biological Chemistry*, 254(12), 5264–5271.
- Bernhardt, R. (2006). Cytochromes P450 as versatile biocatalysts. *Journal of Biotechnology*, 124(1), 128–145.
- Bernhardt, R., & Urlacher, V. B. (2014). Cytochromes P450 as promising catalysts for biotechnological application: Chances and limitations. *Applied Microbiology and Biotechnology*, 98(14), 6185–6203.
- Betancor, L., & Luckarift, H. R. (2010). Co-immobilized coupled enzyme systems in biotechnology. *Biotechnology and Genetic Engineering Reviews*, 27(1), 95–114.

- Bleif, S., Hannemann, F., Lisurek, M., von Kries, J. P., Zapp, J., Dietzen, M., Antes, I., & Bernhardt, R. (2011). Identification of CYP106A2 as a Regioselective Allylic Bacterial Diterpene Hydroxylase. *ChemBioChem*, 12(4), 576–582.
- Bleif, S., Hannemann, F., Zapp, J., Hartmann, D., Jauch, J., & Bernhardt, R. (2012). A new *Bacillus megaterium* whole-cell catalyst for the hydroxylation of the pentacyclic triterpene 11-keto- β -boswellic acid (KBA) based on a recombinant cytochrome P450 system. *Applied Microbiology and Biotechnology*, 93(3), 1135–1146.
- Bornscheuer, U. T. (2003). Immobilizing Enzymes: How to Create More Suitable Biocatalysts. *Angewandte Chemie International Edition*, 42(29), 3336–3337.
- Brandt, B., Hidalgo, A., & Bornscheuer, U. T. (2006). Immobilization of enzymes in microtiter plate scale. *Biotechnology Journal: Healthcare Nutrition Technology*, 1(5), 582–587.
- Bureik, M., Schiffler, B., Hiraoka, Y., Vogel, F., & Bernhardt, R. (2002). Functional expression of human mitochondrial CYP11B2 in fission yeast and identification of a new internal electron transfer protein, etp1. *Biochemistry*, 41(7), 2311–2321.
- Burova, T., Beckert, V., Uhlmann, H., Ristau, O., Bernhardt, R., & Pfeil, W. (1996). Conformational stability of adrenodoxin mutant proteins. *Protein Science*, 5(9), 1890–1897.
- Cao, P.-R., Bu, H., Dumas, B., & Bernhardt, R. (2000). Construction and characterization of a catalytic fusion protein system: P-45011 β -adrenodoxin reductase-adrenodoxin. *Biochimica et Biophysica Acta*, 1476(2), 253–264.
- Cassimjee, K. E. , Kourist, R. , Lindberg, D. , Wittrup Larsen, M. , Thanh, N. H. , Widersten, M. , Bornscheuer, U. T., & Berglund, P. (2011). One-step enzyme extraction and immobilization for biocatalysis applications. *Biotechnology Journal*, 6(4), 463–469.
- Chen, X., Zaro, J. L., & Shen, W.-C. (2013). Fusion protein linkers: Property, design and functionality. *Advanced Drug Delivery Reviews*, 65(10), 1357–1369.
- Cherry, J. R., & Fidantsef, A. L. (2003). Directed evolution of industrial enzymes: an update. *Current Opinion in Biotechnology*, 14(4), 438–443.
- Chiliza, Z. E., Martínez-Oyanedel, J., & Syed, K. (2020). An overview of the factors playing a role in cytochrome P450 monooxygenase and ferredoxin interactions. *Biophysical Reviews*, 12(5), 1217–1222.

- Chun, Y. J., Shimada, T., Sanchez-Ponce, R., Martin, M. v., Lei, L., Zhao, B., Kelly, S. L., Waterman, M. R., Lamb, D. C., & Guengerich, F. P. (2007). Electron transport pathway for a *Streptomyces* cytochrome P450: Cytochrome P450 105D5-catalyzed fatty acid hydroxylation in *Streptomyces coelicolor* A3(2). *Journal of Biological Chemistry*, 282(24), 17486–17500.
- Ciaramella, A., Minerdi, D., & Gilardi, G. (2017). Catalytically self-sufficient cytochromes P450 for green production of fine chemicals. *Rendiconti Lincei*, 28(1), 169–181.
- Coghlan, V. M., & Vickery, L. E. (1992). Electrostatic Interactions Stabilizing Ferredoxin Electron Transfer Complexes. Disruption by “Conservative” Mutations. *Journal of Biological Chemistry*, 267(13), 8932–8935.
- Cojocaru, V., Winn, P. J., & Wade, R. C. (2007). The ins and outs of cytochrome P450s. *Biochimica et Biophysica Acta - General Subjects*, 1770(3), 390–401.
- Correddu, D., di Nardo, G., & Gilardi, G. (2021). Self-Sufficient Class VII Cytochromes P450: From Full-Length Structure to Synthetic Biology Applications. *Trends in Biotechnology*, 39(11), 1184–1207.
- Denisov, I. G., Makris, T. M., Sligar, S. G., & Schlichting, I. (2005). Structure and chemistry of cytochrome P450. *Chemical Reviews*, 105(6), 2253–2277.
- di Nardo, G., & Gilardi, G. (2020). Natural Compounds as Pharmaceuticals: The Key Role of Cytochromes P450 Reactivity. *Trends in Biochemical Sciences*, 45(6), 511–525.
- Dueber, J. E., Wu, G. C., Malmirchegini, G. R., Moon, T. S., Petzold, C. J., Ullal, A. v., Prather, K. L. J., & Keasling, J. D. (2009). Synthetic protein scaffolds provide modular control over metabolic flux. *Nature Biotechnology*, 27(8), 753–759.
- Engström, K., Johnston, E. v, Verho, O., Gustafson, K. P. J., Shakeri, M., Tai, C.-W., & Bäckvall, J.-E. (2013). Co-immobilization of an Enzyme and a Metal into the Compartments of Mesoporous Silica for Cooperative Tandem Catalysis: An Artificial Metalloenzyme. *Angewandte Chemie International Edition*, 52(52), 14006–14010.
- Ewen, K. M., Kleser, M., & Bernhardt, R. (2011). Adrenodoxin: The archetype of vertebrate-type [2Fe-2S] cluster ferredoxins. *Biochimica et Biophysica Acta - Proteins and Proteomics*, 1814(1), 111–125.
- Ewen, K. M., Schiffler, B., Uhlmann-Schiffler, H., Bernhardt, R., & Hannemann, F. (2008). The endogenous adrenodoxin reductase-like flavoprotein arh1 supports heterologous

- cytochrome P450-dependent substrate conversions in *Schizosaccharomyces pombe*. *FEMS Yeast Research*, 8(3), 432–441.
- Fári, M., & Kralovánszky, U. (2006). The founding father of biotechnology: Károly (Karl) Ereky. *International Journal of Horticultural Science*, 12(1), 9–12.
- Gandomkar, S., Dennig, A., Dordic, A., Ucas Hammerer, L., Athias Pickl, M., Haas, T., Hall, M., & Faber, K. (2018). Biocatalytic Oxidative Cascade for the Conversion of Fatty Acids into α -Ketoacids via Internal H_2O_2 Recycling. *Angewandte Chemie International Edition*, 57(2), 427–430.
- Garfinkel, D. (1958). Studies on pig liver microsomes. I. Enzymic and pigment composition of different microsomal fractions. *Archives of Biochemistry and Biophysics*, 77(2), 493–509.
- Gilardi, G., Mehareenna, Y. T., Tsotsou, G. E., Sadeghi, S. J., Fairhead, M., & Giannini, S. (2002). Molecular Lego: design of molecular assemblies of P450 enzymes for nanobiotechnology. *Biosensors & Bioelectronics*, 17(1–2), 133–145.
- Gillam, E. M. J. (2008). Engineering Cytochrome P450 Enzymes. *Chemical Research in Toxicology*, 21(1), 220–231.
- Glerum, D. M., Muroff, I., Jin, C., & Tzagoloff, A. (1997). COX15 Codes for a Mitochondrial Protein Essential for the Assembly of Yeast Cytochrome Oxidase. *The Journal of Biological Chemistry*, 272(30), 19088–19094.
- Gnanasekaran, T., Karcher, D., Nielsen, A. Z., Martens, H. J., Ruf, S., Kroop, X., Olsen, C. E., Motawie, M. S., Pribil, M., Møller, B. L., Bock, R., & Jensen, P. E. (2016). Transfer of the cytochrome P450-dependent dhurrin pathway from *Sorghum bicolor* into *Nicotiana tabacum* chloroplasts for light-driven synthesis. *Journal of Experimental Botany*, 67(8), 2495–2506.
- Goñi, G., Zöllner, A., Lisurek, M., Velázquez-Campoy, A., Pinto, S., Gómez-Moreno, C., Hannemann, F., Bernhardt, R., & Medina, M. (2009). Cyanobacterial electron carrier proteins as electron donors to CYP106A2 from *Bacillus megaterium* ATCC 13368. *Biochimica et Biophysica Acta - Proteins and Proteomics*, 1794(11), 1635–1642.
- Gotoh, O. (1992). Substrate Recognition Sites in Cytochrome P450 Family 2 (CYP2) Proteins Inferred from Comparative Analysis of Amino Acid and Coding Nucleotide Sequences. *The Journal of Biological Chemistry*, 267(1), 83–90.

- Greule, A., Stok, J. E., de Voss, J. J., & Cryle, M. J. (2018). Unrivalled diversity: the many roles and reactions of bacterial cytochromes P450 in secondary metabolism. *Natural Product Reports*, 35(8), 757–791.
- Gricman, Ł., Weissenborn, M. J., Hoffmann, S. M., Borlinghaus, N., Hauer, B., & Pleiss, J. (2016). Redox Partner Interaction Sites in Cytochrome P450 Monooxygenases: In Silico Analysis and Experimental Validation. *ChemistrySelect*, 1(6), 1243–1251.
- Grinberg, A., & Bernhardt, R. (1998). Effect of replacing a conserved proline residue on the function and stability of bovine adrenodoxin. *Protein Engineering*, 11(11), 1057–1064.
- Grinberg, A., Hannemann, F., Schiffler, B., Müller, J., Heinemann, U., & Bernhardt, R. (2000). Adrenodoxin: Structure, Stability, and Electron Transfer Properties. *Proteins: Structure, Function, and Bioinformatics*, 40(4), 590–612.
- Grogan, G. (2011). Cytochromes P450: Exploiting diversity and enabling application as biocatalysts. *Current Opinion in Chemical Biology*, 15(2), 241–248.
- Guengerich, F. P. (2018). Mechanisms of Cytochrome P450-Catalyzed Oxidations. *ACS Catalysis*, 8(12), 10964–10976.
- Hammerer, L., Winkler, C. K., & Kroutil, W. (2018). Regioselective Biocatalytic Hydroxylation of Fatty Acids by Cytochrome P450s. *Catalysis Letters*, 148, 787–812.
- Han, Y., Lee, S. S., & Ying, J. Y. (2006). Pressure-driven enzyme entrapment in siliceous mesocellular foam. *Chemistry of Materials*, 18(3), 643–649.
- Han, Y.-J., Watson, J. T., Stucky, G. D., & Butler, A. (2002). Catalytic activity of mesoporous silicate-immobilized chloroperoxidase. In *Journal of Molecular Catalysis B: Enzymatic* (Vol. 17).
- Hannemann, F., Bichet, A., Ewen, K. M., & Bernhardt, R. (2007). Cytochrome P450 systems-biological variations of electron transport chains. *Biochimica et Biophysica Acta - General Subjects*, 1770(3), 330–344.
- Hannemann, F., Rottmann, M., Schiffler, B., Zapp, J., & Bernhardt, R. (2001). The loop region covering the iron-sulfur cluster in adrenodoxin comprises a new interaction site for redox partners. *Journal of Biological Chemistry*, 276(2), 1369–1375.
- Hannemann, F., Virus, C., & Bernhardt, R. (2006). Design of an Escherichia coli system for whole cell mediated steroid synthesis and molecular evolution of steroid hydroxylases. *Journal of Biotechnology*, 124(1), 172–181.

- Hanukoglu, I. (1992). Steroidogenic enzymes: Structure, function, and role in regulation of steroid hormone biosynthesis. *The Journal of Steroid Biochemistry and Molecular Biology*, 43(8), 779–804.
- Hanukoglu, I., Suh, B. S., Himmelhoch, S., & Amsterdam, A. (1990). Induction and Mitochondrial Localization of Cytochrome P450_{scc} System Enzymes in Normal and Transformed Ovarian Granulosa Cells. *The Journal of Cell Biology*, 111(4), 1373–1381.
- Hasemann, C. A., Kurumbail, R. G., Boddupalli, S. S., Peterson, J. A., & Deisenhofer, J. (1995). Structure and function of cytochromes P450: a comparative analysis of three crystal structures. *Structure*, 3(1), 41–62.
- Hebeda, R. E., Styrlund, C. R., & Teague, W. M. (1988). Benefits of *Bacillus megaterium* Amylase in Dextrose Production. *Starch*, 40(1), 33–36.
- Hirakawa, H., Kakitani, A., & Nagamune, T. (2013). Introduction of Selective Intersubunit Disulfide Bonds into Self-Assembly Protein Scaffold to Enhance an Artificial Multienzyme Complex's Activity. *Biotechnology and Bioengineering*, 110(7), 1858–1864.
- Hirakawa, H., & Nagamune, T. (2013). Use of *Sulfolobus solfataricus* PCNA Subunit Proteins to Direct the Assembly of Multimeric Enzyme Complexes. In *Methods in Molecular Biology* (Vol. 978, pp. 149–163). Humana Press, Totowa, NJ.
- Holden, H. M., Jacobson, B. L., Hurley, J. K., Tollin, G., Oh, B.-H., Skjeldal, L., Chae, Y. K., Cheng, H., Xia, B., & Markley, J. L. (1994). Structure-Function Studies of [2Fe-2S] Ferredoxins. *Journal of Bioenergetics and Biomembranes*, 26(1), 67–88.
- Hollingsworth, S. A., Batabyal, D., Nguyen, B. D., & Poulos, T. L. (2016). Conformational selectivity in cytochrome P450 redox partner interactions. *Proceedings of the National Academy of Sciences of the United States of America*, 113(31), 8723–8728.
- Hudson, S., Cooney, J., & Magner, E. (2008). Proteins in mesoporous silicates. *Angewandte Chemie - International Edition*, 47(45), 8582–8594.
- Ishimura, K., & Fujita, H. (1997). Light and Electron Microscopic Immunohistochemistry of the Localization of Adrenal Steroidogenic Enzymes. *Microscopy Research and Technique*, 36(6), 445–453.
- Ispas, C., Sokolov, I., & Andreescu, S. (2009). Enzyme-functionalized mesoporous silica for bioanalytical applications. *Analytical and Bioanalytical Chemistry*, 393(2), 543–554.

- Jackson, C. J., Lamb, D. C., Marczylo, T. H., Warrilow, A. G. S., Manning, N. J., Lowe, D. J., Kelly, D. E., & Kelly, S. L. (2002). A Novel Sterol 14 α -Demethylase/Ferredoxin Fusion Protein (MCCYP51FX) from *Methylococcus capsulatus* Represents a New Class of the Cytochrome P450 Superfamily. *Journal of Biological Chemistry*, 277(49), 46959–46965.
- Janocha, S., & Bernhardt, R. (2013). Design and characterization of an efficient CYP105A1-based whole-cell biocatalyst for the conversion of resin acid diterpenoids in permeabilized *Escherichia coli*. *Applied Microbiology and Biotechnology*, 97(17), 7639–7649.
- Janocha, S., Carius, Y., Hutter, M., Lancaster, C. R. D., & Bernhardt, R. (2016). Crystal Structure of CYP106A2 in Substrate-Free and Substrate-Bound Form. *ChemBioChem*, 17(9), 852–860.
- Janocha, S., Schmitz, D., & Bernhardt, R. (2015). Terpene hydroxylation with microbial cytochrome P450 monooxygenases. In *Biotechnology of isoprenoids* (Vol. 148, pp. 215–250). Springer Science and Business Media Deutschland GmbH.
- Jenkins, C. M., & Waterman, M. R. (1994). Flavodoxin and NADPH-Flavodoxin Reductase from *Escherichia coli* Support Bovine Cytochrome P450c17 Hydroxylase Activities. *The Journal of Biological Chemistry*, 269(44), 27401–27408.
- Jung, D., Paradiso, M., & Hartmann, M. (2009). Formation of cross-linked glucose oxidase aggregates in mesocellular foams. *Journal of Materials Science*, 44(24), 6747–6753.
- Kang, M. J., Lisurek, M., Bernhardt, R., & Hartmann, R. W. (2004). Use of high-performance liquid chromatography/electrospray ionization collision-induced dissociation mass spectrometry for structural identification of monohydroxylated progesterones. *Rapid Communications in Mass Spectrometry*, 18(23), 2795–2800.
- Kannan, K., Mukherjee, J., & Gupta, M. N. (2014). Immobilization of a Lipase on Mesocellular Foam of Silica for Biocatalysis in Low-water-containing Organic Solvents. *Chemistry Letters*, 43(7), 1064–1066.
- Karasawa, M., Stanfield, J. K., Yanagisawa, S., Shoji, O., & Watanabe, Y. (2018). Whole-Cell Biotransformation of Benzene to Phenol Catalysed by Intracellular Cytochrome P450BM3 Activated by External Additives. *Angewandte Chemie - International Edition*, 57(38), 12264–12269.
- Kazenwadel, F., Franzreb, M., & Rapp, B. E. (2015). Synthetic enzyme supercomplexes: co-immobilization of enzyme cascades. *Analytical Methods*, 7(10), 4030–4037.

- Kelly, S. L., & Kelly, D. E. (2013). Microbial cytochromes P450: Biodiversity and biotechnology. Where do cytochromes P450 come from, what do they do and what can they do for US? *Philosophical Transactions of the Royal Society B: Biological Sciences*, 368(1612), 20120476.
- Khatri, Y., Schiffrin, A., & Bernhardt, R. (2017). Investigating the effect of available redox protein ratios for the conversion of a steroid by a myxobacterial CYP260A1. *FEBS Letters*, 591(8), 1126–1140.
- Kille, S., Zilly, F. E., Acevedo, J. P., & Reetz, M. T. (2011). Regio- and stereoselectivity of P450-catalysed hydroxylation of steroids controlled by laboratory evolution. *Nature Chemistry*, 3(9), 738–743.
- Kim, J., Desch, R. J., Thiel, S. W., Gulianti, V. v, & Pinto, N. G. (2011). Energetics of protein adsorption on amine-functionalized mesostructured cellular foam silica. *Journal of Chromatography A*, 1218, 7796–7803.
- Kimmich, N., Das, A., Sevrioukova, I., Mehareenna, Y., Sligar, S. G., & Poulos, T. L. (2007). Electron Transfer between Cytochrome P450cin and Its FMN-containing Redox Partner, Cindoxin . *The Journal of Biological Chemistry*, 282(37), 27006–27011.
- Kiss, F. M., Lundemo, M. T., Zapp, J., Woodley, J. M., & Bernhardt, R. (2015). Process development for the production of 15 β -hydroxycyproterone acetate using *Bacillus megaterium* expressing CYP106A2 as whole-cell biocatalyst. *Microbial Cell Factories*, 14(1), 1–13.
- Kleser, M., Hannemann, F., Hutter, M., Zapp, J., & Bernhardt, R. (2012). CYP105A1 mediated 3-hydroxylation of glimepiride and glibenclamide using a recombinant *Bacillus megaterium* whole-cell catalyst. *Journal of Biotechnology*, 157(3), 405–412.
- Klingenberg, M. (1958). Pigments of rat liver microsomes. *Archives of Biochemistry and Biophysics*, 75(2), 376–386.
- Kokorin, A., Parshin, P. D., Bakkes, P. J., Pometun, A. A., Tishkov, V. I., & Urlacher, V. B. (2021). Genetic fusion of P450 BM3 and formate dehydrogenase towards self-sufficient biocatalysts with enhanced activity. *Scientific Reports*, 11(1), 1–13.
- Kumar, A., & Singh, S. (2013). Directed evolution: tailoring biocatalysts for industrial applications. *Critical Reviews in Biotechnology*, 33(4), 365–378.

- Kumar, S. (2010). Engineering cytochrome P450 biocatalysts for biotechnology, medicine and bioremediation. *Expert Opinion on Drug Metabolism & Toxicology*, 6(2), 115–131.
- Kumar, S., Jin, M., & Weemhoff, J. L. (2012). Cytochrome P450-Mediated Phytoremediation using Transgenic Plants: A Need for Engineered Cytochrome P450 Enzymes. *Journal of Petroleum & Environmental Biotechnology*, 3(5).
- Lamb, D. C., Follmer, A. H., Goldstone, J. v., Nelson, D. R., Warrilow, A. G., Price, C. L., True, M. Y., Kelly, S. L., Poulos, T. L., & Stegeman, J. J. (2019). On the occurrence of cytochrome P450 in viruses. *Proceedings of the National Academy of Sciences of the United States of America*, 116(25), 12343–12352.
- Leuchs, S., & Greiner, L. (2011). Alcohol Dehydrogenase from *Lactobacillus brevis*: A Versatile Robust Catalyst for Enantioselective Transformations. *Chemical and Biochemical Engineering Quarterly*, 25(2), 267–281.
- Lewis, J. C., Bastian, S., Bennett, C. S., Fu, Y., Mitsuda, Y., Chen, M. M., Greenberg, W. A., Wong, C.-H., & Arnold, F. H. (2009). Chemoenzymatic elaboration of monosaccharides using engineered cytochrome P450 BM3 demethylases. *Proceedings of the National Academy of Sciences of the United States of America*, 106(39), 16550–16555.
- Li, H., & Poulos, T. L. (1997). The structure of the cytochrome P450BM-3 haem domain complexed with the fatty acid substrate, palmitoleic acid. *Nature Structural Biology*, 4(2), 140–146.
- Li, S., Du, L., & Bernhardt, R. (2020). Redox Partners: Function Modulators of Bacterial P450 Enzymes. *Trends in Microbiology*, 28(6), 445–454.
- Li, Z., Jiang, Y., Guengerich, F. P., Ma, L., Li, S., & Zhang, W. (2020). Engineering cytochrome P450 enzyme systems for biomedical and biotechnological applications. *Journal of Biological Chemistry*, 295(3), 833–849.
- Lin, R., Amrute, A. P., & Pérezpérez-Ramírez, J. (2017). Halogen-Mediated Conversion of Hydrocarbons to Commodities. *Chemical Reviews*, 117(5), 4182–4247.
- Lisurek, M., Kang, M. J., Hartmann, R. W., & Bernhardt, R. (2004). Identification of monohydroxy progesterones produced by CYP106A2 using comparative HPLC and electrospray ionisation collision-induced dissociation mass spectrometry. *Biochemical and Biophysical Research Communications*, 319(2), 677–682.

- Lisurek, M., Simgen, B., Antes, I., & Bernhardt, R. (2008). Theoretical and experimental evaluation of a CYP106A2 low homology model and production of mutants with changed activity and selectivity of hydroxylation. *ChemBioChem*, 9(9), 1439–1449.
- Maurer, S. C., Schulze, H., Schmid, R. D., & Urlacher, V. (2003). Immobilisation of P450 BM-3 and an NADP+ Cofactor Recycling System: Towards a Technical Application of Heme-Containing Monooxygenases in Fine Chemical Synthesis. *Advanced Synthesis and Catalysis*, 345(6–7), 802–810.
- McAleer, W. J., Jacob, T. A., Turnbull, L. B., Schoenewaldt, E. F., & Stoudt, T. H. (1958). Hydroxylation of Progesterone by *Bacillus cereus* and *Bacillus megaterium*. *Archives of Biochemistry and Biophysics*, 73(1), 127–130.
- McIntosh, J. A., Farwell, C. C., & Arnold, F. H. (2014). Expanding P450 catalytic reaction space through evolution and engineering. *Current Opinion in Chemical Biology*, 19, 126–134.
- McLean, K. J., Girvan, H. M., & Munro, A. W. (2007). Cytochrome P450/redox partner fusion enzymes: Biotechnological and toxicological prospects. *Expert Opinion on Drug Metabolism and Toxicology*, 3(6), 847–863.
- McRee, D. E., & David, P. R. (1999). *Practical protein crystallography*. Academic Press.
- Meunier, B., de Visser, S. P., & Shaik, S. (2004). Mechanism of oxidation reactions catalyzed by cytochrome P450 enzymes. *Chemical Reviews*, 104(9), 3947–3980.
- Meyer, J. (2000). Clostridial Iron-Sulphur Proteins Fermentation Symposium. *J. Mol. Microbiol. Biotechnol Minireview*, 2(1), 9–14.
- Meyer, J. (2008). Iron-sulfur protein folds, iron-sulfur chemistry, and evolution. *Journal of Biological Inorganic Chemistry*, 13(2), 157–170.
- Miles, C. S., Ost, T. W. B., Noble, M. A., Munro, A. W., & Chapman, S. K. (2000). Protein engineering of cytochromes P-450. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology*, 1543(2), 383–407.
- Miles, J. S., Munro, A. W., Rospendowski, B. N., Smith, W. E., Mcknight, J., & Thomson, A. J. (1992). Domains of the catalytically self-sufficient cytochrome P-450 BM-3. Genetic construction, overexpression, purification and spectroscopic characterization. *Biochemical Journal*, 288(2), 503–509.

- Moser, C. C., Keske, J. M., Warncke, K., Farid, R. S., & Dutton, P. L. (1992). Nature of biological electron transfer. *Nature*, 355(6363), 796–802.
- Müller, A., Müller, J. J., Muller, Y. A., Uhlmann, H., Bernhardt, R., & Heinemann, U. (1998). New aspects of electron transfer revealed by the crystal structure of a truncated bovine adrenodoxin, Adx(4-108). *Structure*, 6(3), 269–280.
- Müller, J. J., Hannemann, F., Schiffler, B., Ewen, K. M., Kappl, R., Heinemann, U., & Bernhardt, R. (2011). Structural and thermodynamic characterization of the adrenodoxin-like domain of the electron-transfer protein Etp1 from *Schizosaccharomyces pombe*. *Journal of Inorganic Biochemistry*, 105(7), 957–965.
- Müller, J. J., Lapko, A., Bourenkov, G., Ruckpaul, K., & Heinemann, U. (2001). Adrenodoxin Reductase-Adrenodoxin Complex Structure Suggests Electron Transfer Path in Steroid Biosynthesis. *Journal of Biological Chemistry*, 276(4), 2786–2789.
- Müller, J. J., Müller, A., Rottmann, M., Bernhardt, R., & Heinemann, U. (1999). Vertebrate-type and Plant-type Ferredoxins: Crystal Structure Comparison and Electron Transfer Pathway Modelling. *Journal of Molecular Biology*, 264(2), 501–513.
- Munro, A. W., Girvan, H. M., & McLean, K. J. (2007). Cytochrome P450-redox partner fusion enzymes. *Biochimica et Biophysica Acta - General Subjects*, 1770(3), 345–359.
- Myers, W. K., Lee, Y.-T., Britt, R. D., & Goodin, D. B. (2013). The Conformation of P450cam in Complex with Putidaredoxin Is Dependent on Oxidation State. *J. Am. Chem. Soc.*, 135(32), 11732–11735.
- Nakamura, Y., Otsuka, H., & Tamauan, B. (1966). Requirement of a new flavoprotein and a non-heme iron-containing protein in the steroid 11 β - and 18-hydroxylase system. *Biochimica et Biophysica Acta - Enzymology and Biological Oxidation*, 122(1), 34–42.
- Nebert, D. W., Nelson, D. R., Adesnik, M., Coon, M. J., Estabrook, R. W., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Kemper, B., Levin, W., Phillips, I. R., Sato, R., & Waterman, M. R. (1989). The P450 Superfamily: Updated Listing of All Genes and Recommended Nomenclature for the Chromosomal Loci. *DNA*, 8(1), 1–13.
- Nelson, D. R. (2009). The Cytochrome P450 Homepage. *Human Genomics*, 4(1), 59–65. <http://drnelson.utmem.edu/>.
- Nelson, D. R. (2018). Cytochrome P450 diversity in the tree of life. *Biochimica et Biophysica Acta - Proteins and Proteomics*, 1866(1), 141–154.

- Nguyen, K. T., Virus, C., Günnewich, N., Hannemann, F., & Bernhardt, R. (2012). Changing the Regioselectivity of a P450 from C15 to C11 Hydroxylation of Progesterone. *ChemBioChem*, 13(8), 1161–1166.
- Nielsen, A. Z., Ziersen, B., Jensen, K., Mü Nter Lassen, L., Olsen, C. E., Lindberg Møller, B., & Jensen, P. E. (2013). Redirecting Photosynthetic Reducing Power toward Bioactive Natural Product Synthesis. *ACS Synthetic Biology*, 2(6), 308–315.
- Nikolaus, J., Nguyen, K. T., Virus, C., Riehm, J. L., Hutter, M., & Bernhardt, R. (2017). Engineering of CYP106A2 for steroid 9 α - and 6 β -hydroxylation. *Steroids*, 120, 41–48.
- Nöth, M., Hussmann, L., Belthle, T., El-Awaad, I., Davari, M. D., Jakob, F., Pich, A., & Schwaneberg, U. (2020). MicroGelzymes: pH-Independent Immobilization of Cytochrome P450 BM3 in Microgels. *Biomacromolecules*, 21(12), 5128–5138.
- Omura, T., Sanders, E., Estabrook, R. W., Cooper, D. Y., & Rosenthal, O. (1966). Isolation from adrenal cortex of a nonheme iron protein and a flavoprotein functional as a reduced triphosphopyridine nucleotide-cytochrome P-450 reductase. *Archives of Biochemistry and Biophysics*, 117(3), 660–673.
- Omura, T., & Sato, R. (1964). The Carbon Monoxide-binding Pigment of Liver Microsomes. I. Evidence for its Hemoprotein Nature. *The Journal of Biological Chemistry*, 239(7), 2370–2378.
- O'Reilly, E., Köhler, V., Flitsch, S. L., & Turner, N. J. (2011). Cytochromes P450 as useful biocatalysts: addressing the limitations. *Chemical Communications*, 47(9), 2490–2501.
- Ortiz de Montellano, P. R. (2005). *Cytochrome P450 Structure, Mechanism, and Biochemistry: Vol. Third Edition*. Kluwer Academic/Plenum Publishers, New York.
- Pandya, P. H., Jasra, R. v, Newalkar, B. L., & Bhatt, P. N. (2005). Studies on the activity and stability of immobilized α -amylase in ordered mesoporous silicas. *Microporous and Mesoporous Materials*, 77(1), 67–77.
- Parajuli, N., Basnet, D. B., Lee, H. C., Sohng, J. K., & Liou, K. (2004). Genome analyses of *Streptomyces peucetius* ATCC 27952 for the identification and comparison of cytochrome P450 complement with other *Streptomyces*. *Archives of Biochemistry and Biophysics*, 425(2), 233–241.
- Peterson, J. A., & Graham, S. E. (1998). A close family resemblance: the importance of structure in understanding cytochromes P450. *Structure*, 6(9), 1079–1085.

- Pikuleva, I. A., Tesh, K., Waterman, M. R., & Kim, Y. (2000). The Tertiary Structure of Full-Length Bovine Adrenodoxin Suggests Functional Dimers. *Archives of Biochemistry and Biophysics*, 373(1), 44–55.
- Pochapsky, S. S., Pochapsky, T. C., & Wei, J. W. (2003). A Model for Effector Activity in a Highly Specific Biological Electron Transfer Complex: The Cytochrome P450 cam- Putidaredoxin Couple. *Biochemistry*, 42(19), 5649–5656.
- Pochapsky, T. C., Kazanis, S., & Dang, M. (2010). Conformational Plasticity and Structure/Function Relationships in Cytochromes P450. *Antioxidants & Redox Signaling*, 13(8), 1273–1296.
- Polic, V., & Auclair, K. (2014). Controlling substrate specificity and product regio- and stereoselectivities of P450 enzymes without mutagenesis. *Bioorganic and Medicinal Chemistry*, 22(20), 5547–5554.
- Poulos, T. L. (1995). Cytochrome P450. *Current Opinion in Structural Biology*, 5(6), 767–774..
- Poulos, T. L. (2003). Cytochrome P450 flexibility. *Proceedings of the National Academy of Sciences of the United States of America*, 100(23), 13121–13122.
- Poulos, T. L. (2005). Structural biology of heme monooxygenases. *Biochemical and Biophysical Research Communications*, 338(1), 337–345.
- Prather, K. L. J., & Martin, C. H. (2008). De novo biosynthetic pathways: rational design of microbial chemical factories. *Current Opinion in Biotechnology*, 19(5), 468–474.
- Putkaradze, N., Kiss, F. M., Schmitz, D., Zapp, J., Hutter, M. C., & Bernhardt, R. (2017). Biotransformation of prednisone and dexamethasone by cytochrome P450 based systems – Identification of new potential drug candidates. *Journal of Biotechnology*, 242, 101–110.
- Putkaradze, N., Litzenburger, M., Abdulmughni, A., Milhim, M., Brill, E., Hannemann, F., & Bernhardt, R. (2017). CYP109E1 is a novel versatile statin and terpene oxidase from *Bacillus megaterium*. *Applied Microbiology and Biotechnology*, 101(23–24), 8379–8393.
- Rauschenbach, R., Isernhagen, M., Noeske-Jungblut, C., Boidol, W., & Siewert, G. (1993). Cloning sequencing and expression of the gene for cytochrome P450meg, the steroid-15 β -monooxygenase from *Bacillus megaterium* ATCC 13368. *Molecular and General Genetics MGG*, 241(1), 170–176.
- Ro, D.-K., Paradise, E. M., Ouellet, M., Fisher, K. J., Newman, K. L., Ndungu, J. M., Ho, K. A., Eachus, R. A., Ham, T. S., Kirby, J., Chang, M. C. Y., Withers, S. T., Shiba, Y.,

- Sarpong, R., & Keasling, J. D. (2006). Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature*, 440(7086), 940–943.
- Rosales Hernández, M. C., Mendieta Wejebe, J. E., Vázquez Alcántara, J. I., Ruvalcaba, R. M., Serrano, L. A. G., & Ferrara, J. T. (2005). Immobilization of cytochrome P-450 on MCM-41 with different silicon/aluminum ratios. *Microporous and Mesoporous Materials*, 80(1–3), 25–31.
- Ruettinger, R. T., & Fulco, A. J. (1981). Epoxidation of unsaturated fatty acids by a soluble cytochrome P-450-dependent system from *Bacillus megaterium*. *Journal of Biological Chemistry*, 256(11), 5728–5734.
- Ruijsenaars, H. J., Sperling, E. M. G. M., Wiegerinck, P. H. G., Brands, F. T. L., Wery, J., & de Bont, J. A. M. (2007). Testosterone 15 β -hydroxylation by solvent tolerant *Pseudomonas putida* S12. *Journal of Biotechnology*, 131(2), 205–208.
- Rylott, E. L., Jackson, R. G., Edwards, J., Womack, G. L., Seth-Smith, H. M., Rathbone, D. A., Strand, S. E., & Bruce, N. C. (2006). An explosive-degrading cytochrome P450 activity and its targeted application for the phytoremediation of RDX. *Nature Biotechnology*, 24(2), 216–219.
- Sabbadin, F., Hyde, R., Robin, A., Hilgarth, E.-M., Delenne, M., Flitsch, S., Turner, N., Grogan, G., & Bruce, N. C. (2010). LICRED: A Versatile Drop-In Vector for Rapid Generation of Redox-Self-Sufficient Cytochrome P450s. *ChemBioChem*, 11(7), 987–994.
- Sadeghi, S. J., Mehareenna, Y. T., Fantuzzi, A., Valetti, F., & Gilardi, G. (2000). Engineering artificial redox chains by molecular “Lego.” *Faraday Discussions*, 116, 135–153.
- Sadeghi, S. J., Tsotsou, G. E., Fairhead, M., Mehareenna, Y. T., & Gilardi, G. (2001). Rational Design of P450 Enzymes for Biotechnology. In *Physics and chemistry basis of biotechnology* (pp. 71–104). Springer, Dordrecht.
- Sagadin, T., Riehm, J. L., Milhim, M., Hutter, M. C., & Bernhardt, R. (2018). Binding modes of CYP106A2 redox partners determine differences in progesterone hydroxylation product patterns. *Communications Biology*, 1(1), 1–9.
- Sakaki, T., Yamamoto, K., & Ikushiro, S. (2013). Possibility of application of cytochrome P450 to bioremediation of dioxins. *Biotechnology and Applied Biochemistry*, 60(1), 65–70.

- Schiffler, B., Bureik, M., Reinle, W., Müller, E. C., Hannemann, F., & Bernhardt, R. (2004). The adrenodoxin-like ferredoxin of *Schizosaccharomyces pombe* mitochondria. *Journal of Inorganic Biochemistry*, 98(7), 1229–1237.
- Schmitz, D., Janocha, S., Kiss, F. M., & Bernhardt, R. (2018). CYP106A2 - A versatile biocatalyst with high potential for biotechnological production of selectively hydroxylated steroid and terpenoid compounds. *Biochimica et Biophysica Acta - Proteins and Proteomics*, 1866(1), 11–22.
- Schmitz, D., Zapp, J., & Bernhardt, R. (2012). Hydroxylation of the triterpenoid dipterocarpol with CYP106A2 from *Bacillus megaterium*. *FEBS Journal*, 279(9), 1663–1674.
- Schmitz, D., Zapp, J., & Bernhardt, R. (2014). Steroid conversion with CYP106A2 - production of pharmaceutically interesting DHEA metabolites. *Microbial Cell Factories*, 13(81), 1–13
- Schumacher, S. D., Hannemann, F., Teese, M. G., Bernhardt, R., & Jose, J. (2012). Autodisplay of functional CYP106A2 in *Escherichia coli*. *Journal of Biotechnology*, 161(2), 104–112.
- Schwede, T., & Peitsch, M. (2008). Computational structural biology: Methods and applications. In *Computational Structural Biology: Methods and Applications* (pp. 1–779). World Scientific Publishing Co.
- Sevrioukova, I. F., & Poulos, T. L. (2011). Structural biology of redox partner interactions in P450cam monooxygenase: A fresh look at an old system. *Archives of Biochemistry and Biophysics*, 507(1), 66–74.
- Sherman, D. H., Li, S., Yermalitskaya, L. v, Kim, Y., Smith, J. A., Waterman, M. R., & Podust, L. M. (2006). The Structural Basis for Substrate Anchoring, Active Site Selectivity, and Product Formation by P450 PikC from *Streptomyces venezuelae*. *Journal of Biological Chemistry*, 281(36), 26289–26297.
- Sibbesen, O., de Voss, J. J., & Ortiz de Montellano, P. R. (1996). Putidaredoxin reductase-putidaredoxin-cytochrome P450(cam) triple fusion protein. Construction of a self-sufficient *Escherichia coli* catalytic system. *Journal of Biological Chemistry*, 271(37), 22462–22469.
- Simgen, B., Contzen, J., Schwarzer, R., Bernhardt, R., & Jung, C. (2000). Substrate binding to 15 β -hydroxylase (CYP106A2) probed by FT infrared spectroscopic studies of the iron

- ligand CO stretch vibration. *Biochemical and Biophysical Research Communications*, 269(3), 737–742.
- Solé, J., Caminal, G., Schürmann, M., Álvaro, G., & Guillén, M. (2019). Co-immobilization of P450 BM3 and glucose dehydrogenase on different supports for application as a self-sufficient oxidative biocatalyst. *Journal of Chemical Technology & Biotechnology*, 94(1), 244–255.
- Sono, M., Roach, M. P., Coulter, E. D., & Dawson, J. H. (1996). Heme-Containing Oxygenases. *Chemical Reviews*, 96(7), 2841–2888.
- Spradlin, J., Lee, D., Mahadevan, S., Mahomed, M., Tang, L., Lam, Q., Colbert, A., Shafaat, O. S., Goodin, D., Kloos, M., Kato, M., & Cheruzel, L. E. (2016). Insights into an efficient light-driven hybrid P450 BM3 enzyme from crystallographic, spectroscopic and biochemical studies. *Biochimica et Biophysica Acta - Proteins and Proteomics*, 1864(12), 1732–1738.
- Sugimoto, H., Shinkyo, R., Hayashi, K., Yoneda, S., Yamada, M., Kamakura, M., Ikushiro, S. I., Shiro, Y., & Sakaki, T. (2008). Crystal structure of CYP105A1 (P450SU-1) in complex with 1 α ,25-dihydroxyvitamin D3. *Biochemistry*, 47(13), 4017–4027.
- Szczebara, F. M., Chandelier, C., Villeret, C., Masurel, A., Bourot, S., Duport, C., Blanchard, S., Groisillier, A., Testet, E., Costaglioli, P., Cauet, G., Degryse, E., Balbuena, D., Winter, J., Achstetter, T., Spagnoli, R., Pompon, D., & Dumas, B. (2003). Total biosynthesis of hydrocortisone from a simple carbon source in yeast. *Nature Biotechnology*, 21(2), 143–149.
- Tanaka, T., Yokoyama, S., & Kuroda, Y. (2006). Improvement of Domain Linker Prediction by Incorporating Loop-Length-Dependent Characteristics. *Peptide Science*, 84(2), 161–168.
- Tripathi, S., Li, H., & Poulos, T. L. (2013). Structural basis for effector control and redox partner recognition in cytochrome P450. *Science*, 340(6137), 1227–1230.
- Uhlmann, H., & Bernhardt, R. (1995). The Role of Threonine 54 in Adrenodoxin for the Properties of Its Iron-Sulfur Cluster and Its Electron Transfer Function. *The Journal of Biological Chemistry*, 270(50), 29959–29966.

- Uhlmann, H., Iametti, S., Vecchio, G., Bonomi, F., & Bernhardt, R. (1997). Pro108 is important for folding and stabilization of adrenal ferredoxin, but does not influence the functional properties of the protein. *European Journal of Biochemistry*, 248(3), 897–902.
- Uhlmann, H., Kraft, R., & Bernhardt, R. (1994). C-terminal Region of Adrenodoxin Affects Its Structural Integrity and Determines Differences in Its Electron Transfer Function to Cytochrome P-450. *The Journal of Biological Chemistry*, 269(36), 22557–22564.
- Valikhani, D., Bolivar, J. M., & Pelletier, J. N. (2021). An Overview of Cytochrome P450 Immobilization Strategies for Drug Metabolism Studies, Biosensing, and Biocatalytic Applications: Challenges and Opportunities. *ACS Catalysis*, 11(15), 9418–9434.
- Vary, P. S. (1994). Prime time for *Bacillus megaterium*. *Microbiology*, 140(5), 1001–1013.
- Vickery, L. E. (1997). Molecular recognition and electron transfer in mitochondrial steroid hydroxylase systems. *Steroids*, 62(1), 124–127.
- Vihinen, M., & Mantsiila, P. (1989). Microbial Amylolytic Enzyme. *Critical Reviews in Biochemistry and Molecular Biology*, 24(4), 329–418.
- Virus, C., Lisurek, M., Simgen, B., Hannemann, F., & Bernhardt, R. (2006). Function and engineering of the 15 β -hydroxylase CYP106A2. *Biochemical Society Transactions*, 34(6), 1215–1218.
- von Bühler, C., Le-Huu, P., & Urlacher, V. B. (2013). Cluster Screening: An Effective Approach for Probing the Substrate Space of Uncharacterized Cytochrome P450s. *ChemBioChem*, 14(16), 2189–2198.
- Weber, E., Sirim, D., Schreiber, T., Thomas, B., Pleiss, J., Hunger, M., Gläser, R., & Urlacher, V. B. (2010). Immobilization of P450 BM-3 monooxygenase on mesoporous molecular sieves with different pore diameters. *Journal of Molecular Catalysis B: Enzymatic*, 64(1–2), 29–37.
- Wichmann, R., & Vasic-Racki, D. (2005). Cofactor regeneration at the lab scale. In *Advances in Biochemical Engineering/Biotechnology*, 92, 225–260.
- Yano, J. K., Koo, L. S., Schuller, D. J., Li, H., Ortiz De Montellano, P. R., & Poulos, T. L. (2000). Crystal Structure of a Thermophilic Cytochrome P450 from the Archaeon *Sulfolobus solfataricus*. *Journal of Biological Chemistry*, 275(40), 31086–31092.

- Zhang, W., Du, L., Li, F., Zhang, X., Qu, Z., Han, L., Li, Z., Sun, J., Qi, F., Yao, Q., Sun, Y., Geng, C., & Li, S. (2018). Mechanistic Insights into Interactions between Bacterial Class I P450 Enzymes and Redox Partners. *ACS Catalysis*, 8(11), 9992–10003.
- Zhang, W., Liu, Y., Yan, J., Cao, S., Bai, F., Yang, Y., Huang, S., Yao, L., Anzai, Y., Kato, F., Podust, L. M., Sherman, D. H., & Li, S. (2014). New reactions and products resulting from alternative interactions between the P450 enzyme and redox partners. *Journal of the American Chemical Society*, 136(9), 3640–3646.
- Zhao, F., Bai, P., Liu, T., Li, D., Zhang, X., Lu, W., & Yuan, Y. (2016). Optimization of a cytochrome P450 oxidation system for enhancing protopanaxadiol production in *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering*, 113(8), 1787–1795.
- Ziegler, G. A., & Schulz, G. E. (2000). Crystal Structures of Adrenodoxin Reductase in Complex with NADP⁺ and NADPH Suggesting a Mechanism for the Electron Transfer of an Enzyme Family. *Biochemistry*, 39(36), 10986–10995.
- Ziegler, G. A., Vonnrhein, C., Hanukoglu, I., & Schulz, G. E. (1999). The Structure of Adrenodoxin Reductase of Mitochondrial P 450 Systems: Electron Transfer for Steroid Biosynthesis. *Journal of Molecular Biology*, 289(4), 981–990.
- Zilly, F. E., Acevedo, J. P., Augustyniak, W., Deege, A., Häusig, U. W., & Reetz, M. T. (2011). Tuning a P450 Enzyme for Methane Oxidation. *Angewandte Chemie International Edition*, 50(12), 2720–2724.

5 Abbreviations

15 β -OH-prog 15-beta-hydroxyprogesterone

Å Ångström

aa Amino acid

AdR Adrenodoxin reductase

Adx Adrenodoxin reductase

Arh1 Adrenodoxin reductase homologue 1

ATCC *American Type Culture Collection*

BM3 CYP102A1

BMBF Bundesministerium für Bildung und Forschung (Federal Ministry of Education and Research)

BmCPR *Bacillus megaterium* cytochrome P450 reductase

CO Carbon monoxide

CPR Cytochrome P450 reductase

CYP Cytochrome P450 (or P450)

DOC 11-deoxycorticosterone

Etp1 Electron transfer protein 1

Etp1fd Electron transfer protein 1, ferredoxin domain

FAD Flavine adenine dinucleotide

FeS cluster Iron–sulfur cluster

FMN Flavine mononucleotide

Fpr Ferredoxin-/Flavodoxin reductase from *Escherichia coli*

G6PDH Glucose-6-phosphate-dehydrogenase

His-tag Polyhistidine-tag

HPLC High performance liquid chromatography

k_{app} Apparent rate constant

kDa (or kD)	kilo Dalton
LbADH	Alcohol-dehydrogenase from <i>Lactobacillus brevis</i>
MCF	Mesostructured cellular foam
NADPH	Nicotinamide adenine dinucleotide (phosphate), reduced form
P450	Cytochrome P450 (or CYP)
Pdx	Putidaredoxin
PdR	Putidaredoxin reductase
Rpm	Rotations per minute
RSS	Reichstein's Substance S
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SRS	Substrate recognition site
Strep-tag	Peptide that binds to Strep-tactin
YkuN	Flavodoxins from <i>Bacillus subtilis</i>
YR-P5	Fusion protein of YkuN and Fpr linked by a polyproline linker of 5 repetitions

6 List of figures

Figure 1: General reaction of cytochrome P450 systems	11
Figure 2: Multi-step catalytic cycle of P450s and shunt pathway (Denisov et al., 2005)	12
Figure 3: Overview of the two most common classes of redox systems in P450s (modified from (Hannemann et al., 2007)), A: class I containing three proteins being FAD-containing reductase, ferredoxin as electron shuttle, and the P450 (all soluble in bacterial systems); B class I containing three proteins being FAD-containing reductase and the P450 (both bound to mitochondrial membrane in eucaryotes) as well as the soluble ferredoxin as electron shuttle; C: class II containing two proteins being the P450 and the CPR (both bound to the ER membrane)	16
Figure 4: Electron transport in the steroid hydroxylase system of <i>B. megaterium</i> (Berg et al., 1976))	17
Figure 5: Comparison of overall product yield (blue bars) and main product (15 β -OH-prog, orange bars) respective to the used ferredoxin/flavodoxin	73
Figure 6: AdR-proline linker-LbADH fusion protein model (screenshot). On the left is AdR (461 amino acids), in the middle is the proline linker (23 amino acids), and on the right is LbADH (252 amino acids).	83
Figure 7: TEM images of MCF 13 with 20 nm cells (left) and MCF 16 with 30 nm cells (right). The scale bars are 50 nm.	88
Figure 8: Progesterone conversion (%) by free CYP106A2 in solution or immobilized on MCF-25COO-Co after 10 minutes of reaction time. Black columns represent 15 β -hydroxyprogesterone, diagonally striped columns polyhydroxylated progesterone, and the horizontally striped columns the combined 6 β -, 9 α , and 11 α -hydroxyprogesterone. Adx4-108 and AdR, both in solution, were added to complete the electron transfer chain. For the sufficient supply of electrons, a glucose-6-phosphate dehydrogenase-based NADPH-regenerating system was used.	89