Engineering DoxA activity through the manipulation of associated electron transport partners

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Master's thesis

Abstract

Doxorubicin (DXR) is an anthracycline produced by some strains of the bacterium *Streptomyces peucetius* subsp. *caesius.* It is one of the most commonly used drugs in the treatment of several different types of cancer. It is produced as a result of a complicated biosynthetic pathway. The cytochrome P450 enzyme (CYP450) DoxA is responsible for the final three reaction steps in the biosynthesis. The final step, hydroxylation of daunorubicin (DNR) to DXR, is much less efficient than the preceding two steps, which causes low amounts of DXR to be produced in relation to DNR. This makes large-scale production of DXR difficult and a way to achieve complete biosynthetic conversion of DNR to DXR would be hugely beneficial. This thesis project aimed to find ways to improve DXR biosynthesis by studying the effect of different redox systems on the activity of DoxA. Preliminary data showed that overproduction of DXR might be related to the expression of the ferredoxins X4 and X5. In addition to these, the proteins SFX, SFR, PDX and PDR from other organisms, as well as a fusion of the proteins YkuN and Fpr, and finally, a fusion of DoxA and an RhF-domain, were used to try to improve DoxA activity. The proteins were produced in *Escherichia coli*, and the proteins were purified by affinity chromatography. In vitro activity assays with DoxA and the redox proteins were done to measure the activity of DoxA. The proteins from *S. peucetius* did not cause any activity at all, leading to the conclusions that incompatible pairs had probably been chosen. The unnatural redox systems supported DoxA activity to different extents, with PDX and PDR being the best system identified. Several functioning redox systems that can be used in future research were established in this project, and they all provide valuable insights into redox engineering as a whole and as a way to improve the biosynthesis of DXR.

Keywords: biosynthesis, cytochrome P450, DoxA, doxorubicin, fusion proteins, redox engineering, redox proteins, *Streptomyces peucetius* subsp. *caesius*

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List of abbreviations

BGC	Biosynthetic gene cluster
CYP450	Cytochrome P450
CDR	Cytochrome P450 reductase
DHD	13-dihydrodaunorubicin
DNR	daunorubicin
DOD	13-deoxydaunorubicin
DXR	Doxorubicin
EDTA	Ethylenediaminetetraacetic acid
FAD	Flavin adenine dinucleotide
FDR	Ferredoxin reductase
FDX	Ferredoxin
FLD	Flavodoxin reductase
FMN	Flavin mononucleotide
GDH	Glucose dehydrogenase
HPLC	High-performance liquid chromatography
NAD^+	Nicotinamide adenine dinucleotide, oxidized
NADH	Nicotinamide adenine dinucleotide, reduced
$NADP^+$	Nicotinamide adenine dinucleotide phosphate, oxidized
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
PDR	Putidaredoxin reductase
PDX	Putidaredoxin
PKS	Polyketide synthase
ROS	Reactive oxygen species

SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
SFR	Spinach ferredoxin reductase
SFX	Spinach ferredoxin
TAE	Tris-acetic acid-EDTA

1. Introduction

Cancer is one of the leading causes of death in the modern world, and the increasing rates of chemical exposure and higher life expectancy are causing a continuous increase in the incidence of cancer. Cancer causes immeasurable suffering and grief and is also a huge cost to society. Vast sums of money are spent treating cancer patients, and the development of new treatments is highly expensive (Bray et al., 2018). Because of all this, effective and cost-efficient treatment of cancer has become a goal that motivates a large portion of the research done within the life sciences. In this search for treatments, nature has proven to be a valuable resource of antimicrobial and anticancer compounds. At the turn of the millennium, over 60% of clinically used anticancer agents originated in nature. This means that the compounds are either produced by some organism or that a lead compound found in nature has been improved to meet clinical standards (Cragg, Newman, 2005). The anthracycline compound doxorubicin (DXR) is one such compound, produced by mutants of *Streptomyces peucetius* subsp. *caesius*. It is an old drug, but it is still useful clinically and an interesting research target (Arcamone et al., 1969)

Since nature is full of clinically useful resources, it is of great interest to be able to utilize those resources for the good of society. Bacteria can be used as factories to produce drugs and various enzymes can often carry out complicated chemistry that is challenging to copy synthetically. Taking inspiration from nature and then improving on those concepts is a cornerstone of modern life sciences and the process of doing this is known as bioengineering. The field of bioengineering is an exciting and developing field that is used for many purposes like producing genetically engineered crops, creating bacteria that digest plastic, or utilizing enzymes in industrial scale production of materials. In the field of biochemistry, bioengineering often takes the form of manipulating proteins, which is known as protein engineering. Protein engineering can mean many things, for example changing the amino acid sequence of a protein to make it more stable or give it new functionalities (Li, C. et al., 2020). In the case of DXR, protein engineering has been focused on efforts to make the biosynthesis of DXR more viable, but it could also be used to achieve biosynthesis of similar molecules that do not have the cardiotoxicity-issues of DXR, like the new

compound dimethyldoxorubicin. (Qiao et al., 2020). In this thesis project, a subcategory of protein engineering known as redox engineering will be explored to attempt to improve the biosynthesis of DXR. Redox protein engineering is useful in that it does not require any changes to the sequence of any protein, but rather changes to the interactions between the proteins that supply enzymes with electrons. While redox engineering has been researched in many different contexts, it has yet to be extensively investigated as a potential way to achieve improved biosynthesis of DXR.

2. Literature review

2.1. *Streptomyces* and secondary metabolism

Streptomyces is a genus of gram-positive bacteria belonging to the phylum of Actinobacteria. Species of the genus *Streptomyces* are found ubiquitously in soil samples from anywhere on the planet. Streptomycetes are important for the breakdown of organic matter from plants and the formation of soil; in fact, the evolutionarily ancient bacteria are thought to have been integral in the formation of primeval soil when land-inhabiting plants first started inhabiting the planet (Chater et al., 2010). Species of the genus have many remarkable characteristics that make them very interesting from a research perspective. They are morphologically complex compared to many other bacteria, forming a network of filamentous, fungus-like hyphae in the growth substrate, which then branch off into aerial hyphae. At the tips of the aerial hyphae sporulation happens, allowing spores to be spread and new nutrient sources to be found upon depletion of the old source (Procópio et al., 2012).

The most clinically significant property of streptomycetes is their remarkable ability to produce several biologically active compounds as secondary metabolites. Secondary metabolites differ from primary metabolites in that they are not necessary for the normal growth of the organism but rather give the organism an advantage in competing with its surroundings. Streptomycetes are the origin of the majority of clinically used antibiotics, as well as many antiparasitic and antifungal compounds. In addition to these, several cytotoxic compounds with anti-tumor properties have been identified (Procópio et al., 2012). The numerous genes coding for proteins relevant to the production of a secondary metabolite are commonly clustered together in the genome, in what is known as biosynthetic gene clusters (BGCs). Regulatory proteins and self-resistance proteins (e.g. proteins transporting the secondary metabolite out of the cell) are often coded for by genes in the BGC, in addition to the enzymes that perform the biosynthesis of the secondary metabolite (Cimermancic et al., 2014).

2.2. Anthracyclines and doxorubicin

One of the anticancer compounds derived from streptomycetes is the anthracycline DXR, also known by its trade name adriamycin. DXR was first discovered as a compound produced by a mutant strain of *Streptomyces peucetius* subsp. *caesius*. *S. peucetius* subsp. *caesius* was already known to be a producer of daunorubicin (DNR), the immediate precursor of DXR. Both DNR and DXR have been approved for clinical use (Paul et al., 1989), but DXR was proven to have superior pharmacological properties early on (Arcamone et al., 1969). DXR-producing strains generally produce low ratios of DXR compared to DNR, and the two compounds are difficult to separate. This is one of the main issues in the production of DXR (Weiss, 1992).

Today, DXR is one of the most common chemotherapeutic drugs, used as a first-choice therapy in the treatment of different types of breast cancer, lymphomas, sarcomas and more (Weiss, 1992). DXR is an old drug, but plenty of research is still done on it, and over the years new ways of administering DXR have been adopted. Nowadays it is often administered encapsulated in liposomes, which improves its delivery (Waterhouse et al., 2001). DXR works by intercalating DNA and thereby hindering replication and transcription. At the same time, it acts as a topoisomerase II poison, which leads to double stranded breaks in DNA (Thorn et al., 2011). Formation of reactive oxygen species in a metal-dependent manner is also thought to be an important biological effect of DXR. Newer research also points to DXR causing chromatin damage through histone eviction. The combination of this damage causes cells to undergo apoptosis, and due to the increased reliance on transcription and translation in the rapidly dividing cancer cells, they are affected to a greater extent than healthy cells (Pang et al., 2013)(Tacar, Sriamornsak & Dass, 2013). Due to the extensive damage to cells caused by DXR, patients often experience severe side effects. The most severe of these is cumulative cardiotoxicity, which occurs in many patients and causes problems like arrhythmia and left ventricle dysfunction. The cumulative nature of the cardiotoxicity limits the number of treatments with DXR that can be administered (McGowan et al., 2017).

DXR belongs to the anthracycline class of compounds. Anthracyclines consist of two main parts: a four-ringed polyketide aglycone and a glycan attached at C-7 of the

aglycone. The enzymes responsible for DXR biosynthesis (Figure 1) are found in a single BGC. Several rounds of condensation of 9 malonyl-CoA units and 1 propionyl-CoA unit are carried out by a group of enzymes called polyketide synthases (PKSs). The polyketide formed by these reactions undergoes further modifications to form ε-rhodomycinone. The glycan of DXR, thymidine diphosphate-L-daunosamine, is created through modification of D-glucose-1-phosphate by a group of proteins also coded for in the BGC. The glycan is attached to ε-rhodomycinone, whereafter the anthracycline undergoes further modifications, resulting in the compound 13-deoxydaunorubicin (DOD). The last three steps of the biosynthesis are catalyzed by the cythochrome P450 (CYP450) enzyme DoxA. DoxA hydroxylates DOD at C-13, creating the compound 13-dihydrodaunorubicin (DHD), which is again hydroxylated at C-13 to create DNR. DNR is hydroxylated by DoxA at C-14, creating DXR (Niraula et al., 2010)(Dickens, Priestley & Strohl, 1997).

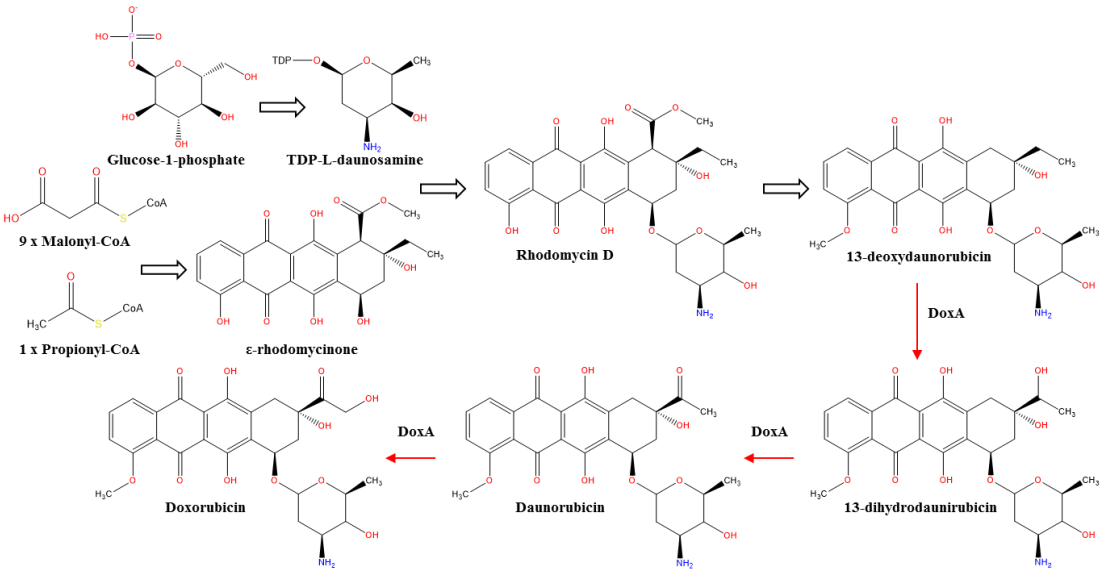

Figure 1: Abbreviated biosynthetic scheme of DXR and its immediate precursors. Thick arrows represent multiple reaction steps.

2.3. Protein engineering and CYP450 enzymes

CYP450 enzymes are a class of enzymes that are of great importance from both a clinical and a synthetic biology perspective (Li, Z. et al., 2020). The name is derived

from the characteristic absorbance peak at 450 nm, which can be observed when doing spectral analysis of reduced, CO-bound CYP450s. The CYP450 superfamily is found across all kingdoms of life and it consists mainly of monooxygenase enzymes that transfer an oxygen atom from heme-bound molecular oxygen to its substrate. These reactions have many different outcomes, including hydroxylation, epoxidation, demethylation, and many others, depending on the specific CYP450 and its substrate. These kinds of reactions can be very difficult and expensive to perform chemically in the synthesis of drugs and other useful molecules, which makes the use of CYP450 enzymes in larger scale chemical synthesis very appealing. CYP450s are reliant on electron transport partners in order to perform their catalytic functions (Girvan, Munro, 2016).

Traditionally, CYP450s have been categorized into two groups: class I and class II systems depending on what kind of redox partner system they utilize. Class I systems (Figure 1A) are typically found in prokaryotes and consist of soluble ferredoxin (FDX) and ferredoxin reductase (FDR) proteins or sometimes flavodoxin (FLD) and flavodoxin reductase proteins (Figure 1C). FDRs contain a flavin adenine dinucleotide (FAD) cofactor, which is reduced when the FDR oxidizes its substrate, NADH or NADPH. FDR shuttles the abstracted electrons further along the transport chain to iron—sulfur clusters in the FDXs. Iron—sulfur clusters come in many variations, with different arrangements of the atoms and different surrounding protein structures. Common examples are the [2Fe—2S]-clusters and [4Fe—4S]-clusters, coordinated by 4 surrounding cysteine residues. FDX transports the electrons to the heme iron of the CYP450, which is then ready to perform its catalytic duties. In flavodoxins the iron—sulfur cluster is replaced by a flavin mononucleotide (FMN) cofactor (Girvan, Munro, 2016)(Bruschi, Guerlesquin, 1988).

The majority of eukaryotic CYP450 enzymes are membrane bound and categorized as class II systems (Figure 1B). They are most commonly found attached to the endoplasmic reticulum via an N-terminal anchor. In order to carry out catalysis, they are reliant on a single redox partner: a membrane bound CYP 450 reductase (CDR) which contains FAD and FMN cofactors and receives electrons from NADH or NADPH (Girvan, Munro, 2016).

More recently, prokaryotic CYP450s with redox partner systems that do not fit into the class I/II paradigm have been discovered. The first one to emerge was P450BM3 from *Bacillus megaterium,* in which a CDR-like domain containing FAD and FMN is fused to the soluble CYP450 (Figure 1D). P450BM3 is not reliant on any further redox partners (Narhi, Fulco, 1986). Another similar fusion protein was found in *Rhodococcus sp.* NCIMB 9784, where the enzyme P450RhF was found to consist of a CYP450-domain and the C-terminally fused RhF-domain, which contains binding sites for NADPH and FMN, as well as a [2Fe—2S]-like cluster similar to the ones found in ferredoxins (Figure 1E). This fusion protein is also self-sufficient and does not require any other redox partners (Nodate, Kubota & Misawa, 2006).

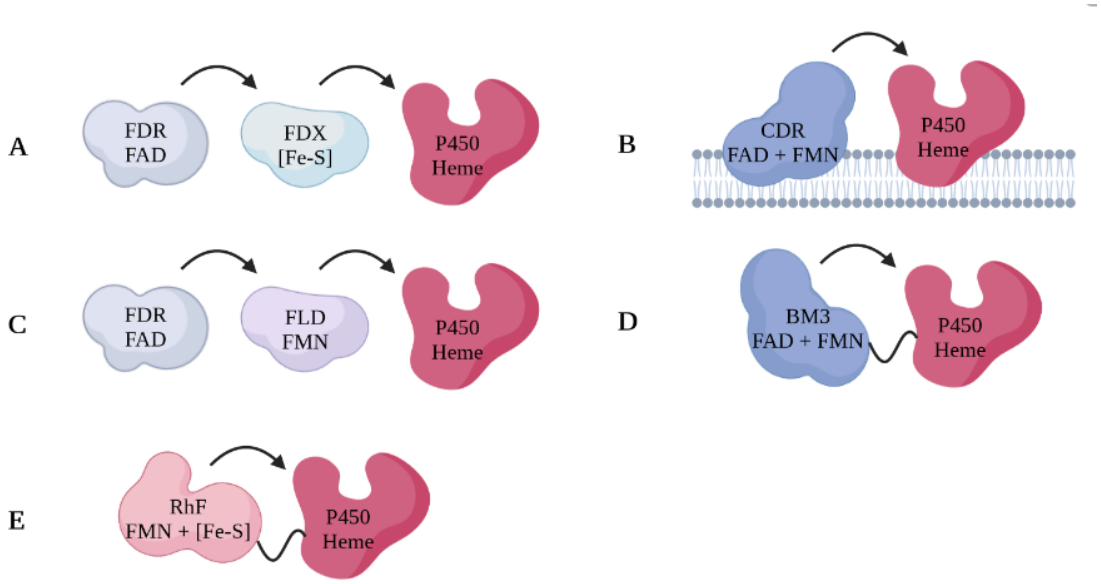

Figure 2: Schematic of different CYP450-associated redox systems, and the cofactors used by the proteins. Arrows represent the flow of electrons. (A) Class I bacterial system, (B) Class II eukaryotic system, (C) flavodoxin-utilizing system, (D) P450BM3-system, (E) P450RhF-system.

2.4. DoxA

DoxA is a class I CYP450, but the associated redox partners have not been identified. No redox proteins are found in the DXR BGC either. However, several putative FDX and FDR genes can be found elsewhere in the *S. peucetius* genome and it is very likely that some combination of them is responsible for transporting electrons to DoxA (Thuan et al., 2018). In the preliminary work conducted before this thesis project,

transcriptomics data of DXR-overproducing strains was obtained via RNA-sequencing. These data pointed to a few electron carrier candidates. The putative ferredoxins X4 and X5 both showed upregulation correlating with the production of DXR over DNR. There was also a connection to the upregulation of proteins that are important in the assembly of Fe—S-clusters and the intake of organic sulfur. Homology of X4 and X5 to ferredoxins from *Pseudomonas putida* pointed to the putative ferredoxin reductases R4 and R7 as likely candidates to work with X4 and X5 (unpublished). There are no available 3D structures of DoxA or the putative redox proteins, which makes it difficult to make structure-based predictions of the interactions.

DoxA can perform hydroxylation on three different substrates: DOD, DHD, and DNR. Its catalytic efficiency varies based on the substrate, with DOD exhibiting the highest efficiency with a k_{cat}/K_m of 22000 ± 1000 $M^{-1}s^{-1}$. DHD has a catalytic efficiency of 14000 ± 2000 $M^{-1}s^{-1}$ and DNR has the worst efficiency by far at 130 ± 10 $M^{-1}s^{-1}$. This gives insight into why DXR producing strains are rare and why DXR is usually produced in relatively small amounts when compared to DNR. DXR also has an inhibitory effect on DoxA, which further complicates *in vivo* production, since DXR needs to be efficiently transported out of the cell (Walczak et al., 1999).

2.5. CYP450 redox engineering

Given their potential usefulness in synthetic biology applications, CYP450s are subject to a lot of research to find ways to engineer their functions. Efforts to engineer the enzymes use many different approaches, like directed evolution, rational design and redox partner engineering (Li et al., 2020).

The versatility of redox systems leaves room for many possible ways to utilize them in engineering CYP450 activity and redox engineering of CYP450 is indeed a broad term. To better understand the implications of redox engineering, a closer look at the mechanisms and interactions of the relevant proteins is required. The transport of electrons from a source to the target protein is not as simple as electrons transferring

from one cofactor to another, but rather a result of transient protein—protein interactions, first between the ferredoxin reductase and the ferredoxin, and then between the ferredoxin and the CYP450 enzyme. The interactions allow the proteins to take on the conformations required for electron transfer. In physiological conditions, the rate-limiting step is often the transfer of electrons from the ferredoxin to the CYP450. The quality of the interactions between proteins is therefore an important part of determining how well a certain set of redox proteins works with a certain P450. The protein—protein interactions can also cause conformational changes that affect the properties of a CYP450, which makes it possible to use redox engineering to affect substrate selectivity (Li, S., Du & Bernhardt, 2020). Using a foreign ferredoxin in a reaction, for example, can have an allosteric effect on the CYP450 and cause changes in its active site, leading to new substrates being able to fit into the active site. Similarly, introducing a new substrate that binds the CYP450 with a different orientation can cause the CYP450 to perform new chemistry and catalyze a reaction that it would not naturally catalyze. In addition to all this, it is important to keep in mind that electron transport *in vivo* does not happen in a vacuum with only one kind of reductase and one type of ferredoxin present. Most organisms have genes coding for several different redox proteins and in many cases different redox proteins are present and able to reduce a CYP450 at the same time. This can possibly be a way for cells to modulate the function of a CYP450 by regulating the expression of redox proteins. To summarize all this, one can say that a CYP450-reaction is a sum of interactions between CYP450, redox proteins, substrate and surroundings. Changing one part of this equation can change the whole reaction, and this makes redox engineering an interesting approach to CYP450 engineering (Li, Du & Bernhardt, 2020).

In practice, there are a few well-established methods used in redox engineering. A commonly used one is to fuse the CYP450 in question to an RhF- or BM3-domain in an effort to create a self-sufficient protein. This can assure efficient transfer of electrons to the heme and reduces the number of proteins that need to be produced. In addition, it can also cause new chemistry to be performed by the CYP450 (Zhang et al., 2014). There is no guarantee that native redox proteins, or even redox proteins expressed in the same operon as a certain CYP450, are more effective at reducing that CYP450 than some redox proteins from a different organism. It is therefore common

practice in redox engineering to exchange native redox partners for other enzymes known to reduce CYP450-enzymes promiscuously (Zhang et al., 2018). A third method used in redox engineering is creating fusion proteins of soluble FDX and FDR proteins (Li et al., 2020). This can allow for effective electron transfer with fewer proteins needing to be purified (Zhang et al., 2018)

Examples of promiscuous redox systems are putidaredoxin (PDX) and putidaredoxin reductase (PDR) from the bacterium *Pseudomonas putida*, SFX and SFR from *Spinacia oleracea* (spinach), the flavodoxin YkuN from *Bacillus subtilis*, and the flavodoxin/ferredoxin reductase Fpr from *Escherichia coli*. These redox pairs have been shown to work for a variety of different CYP450s (Duan et al., 2016), (Girhard et al., 2010) and the PDX-PDR pair has even been used in *in vitro* reactions with DoxA (Rimal et al., 2015). When fusions of redox partners were investigated using the YkuN-Fpr pair with different types of peptide linkers it was found that a YkuN-Fpr construct with a rigid P5 linker $([E/L]PPPP)_5$ was very efficient and worked with several different CYP450-enzymes (Bakkes et al., 2017).

An example of differential, redox partner-dependent product composition was found by Zhang et al. 2014. In this publication, the researchers managed to create new mycinamycin compounds by changing the redox system used by the CYP450 MycG, originating in the related actinomycete *Micromonospora griseorubida*. The physiological redox partners of MycG are unknown, but FDX and FDR from *Spinacea oleracea* (Spinach) as well as fusion to RhF has been used *in vivo* to reconstitute its natural activity of hydroxylating and epoxidating the precursors of the terminal compounds M-I and M-II. However, it turned out that adding the RhF-domain in soluble, non-fused form led to MycG also performing a novel demethylation reaction, leading to the product dMe-M-IV. For further testing, a new domain was constructed, where the [2Fe—2S]-domain of RhF was replaced with that of the spinach ferredoxin. When this RhF-SFX-domain was fused to MycG, it resulted in a similar production profile as the MycG-RhF fusion, albeit with lower rates of conversion. Free RhF-Fdx and MycG, on the other hand, caused even more demethylation than MycG with RhF. This serves as an example of how not just the redox proteins themselves, but also the mode of interaction can have an effect on the function of CYP450 enzymes (Zhang et al., 2014).

Another example of successful CYP450-engineering was done in a strain of the fungus *Penicillum chrysogenum* to produce the important cholesterol-lowering drug pravastatin. The BGC for compactin, a precursor of pravastatin, was cloned into *P. chrysogenum* along with CYP105AS1 from *Amycolatopsis orientalis*. The engineered strain produced the biologically inactive 6-*epi*-pravastatin, which has pharmacologically incorrect stereochemistry. Two rounds of random mutagenesis and screening provided a strain that produced pravastatin almost exclusively. In addition to this, CYP105AS1 had been fused to the RhF-domain from *Rhodococcus sp*., which allowed it to function without any further redox partners. The final optimized strain produced up to 6 g/l of pravastatin in fermentation trials (McLean et al., 2015). This example shows the successful use of both redox partner engineering and directed evolution in using CYP450s as biocatalysts for clinically important synthetic biology.

3. Aims

The project approaches the issue of redox engineering of DoxA from several sides and can be split up into a few separate aims that form the overarching aim. Since it has been shown that the functions of P450 enzymes are significantly affected by the present electron transport pathways, it is likely that the activity of DoxA can also be affected by introducing new redox proteins and artificial systems for electron transport. Non-natural redox systems include fusions of associated proteins and redox proteins from other organisms. While the unnatural redox systems can give valuable insights into the functions of DoxA, the most interesting and significant part of the project is still determining how the putative redox proteins native to *S. peucetius* work with DoxA. If some of the proteins turn out to be important in driving the biosynthetic reaction all the way DXR, it is of great interest to characterize those proteins further. Characterization will give further insight into how the proteins work and place them in a context where they can more easily be compared to other known redox proteins, while simultaneously confirming that they are functional and have been purified properly. Non-natural redox systems can similarly be characterized in order to gain insights into how they might be used in the process of engineering DoxA and to confirm their authenticity.

When these partial aims are put together, they form the overarching aim of gaining new insights that might help achieving efficient biosynthesis of DXR *in vivo*. This is where the real significance of the project lies, and the ideal outcome would be if some of the natural redox proteins were found to be highly effective in helping DoxA synthesize DXR. If this is shown, then the expression of those proteins can be regulated in an attempt to create a superior DXR-producing strain.

The aims outlined above can be summarized as follows:

- To find out how different redox systems affect the catalytic activity and substrate selectivity of DoxA.
- To characterize natural redox proteins X4/X5 and R4/R7 from *S. peucetius*.
- To gain insights that might help improve DXR synthesis *in vivo*.

4. Materials and methods

4.1. Constructs and cloning

The expression and purification of protein is central for this research. DoxA needs to be produced in a manner that results in pure and stable protein, and all the redox partners need to be readily modifiable in order to optimize reaction conditions and yield maximally efficient proteins. Table 1 gives an overview of the synthetic genes used as a starting point for creating constructs for all the proteins. All genes were codon optimized for *E. coli* and ordered as synthetic DNA strands from ThermoScientific.

Table 1: Primary constructs used for protein production

Construct	Linker	His-tag
YkuN-Fpr	P5	N/C
X4R4	PG	N/C
X5R7	P5	N/C
PDX	-	N
PDR	-	N
DoxA	-	N
RhF	Native	N

The constructs were cloned by cleavage at BglII and HindII restriction sites into the plasmid vector pBADHisBΔ. Three types of linkers were used: P5 (GTEPPPPEPPPPLPPPPLPPPPLPPPPAE), which is a rigid linker, the flexible PG-linker (GTEPPPPEPPPPLGGGGSPPPPLPPPPAAV (Bakkes et al., 2017), and the native linker of the RhF-domain (VLHRHQPVTIGEPAAR) (Nodate, Kubota & Misawa, 2006). The linkers could be exchanged through digestion with KpnI and PstI

restriction enzymes if one linker proved to be superior. The ferredoxin/flavodoxin parts of the fusion proteins could be isolated and expressed separately through BglII + KpnI digestion and PstI and HindIII could be used to do the same with the reductase proteins. These restriction enzymes were also used to create fusion proteins with new combinations of proteins, in addition to those laid out in Table 1. The constructs YkuN-Fpr, X4R4 and X5R7 all have a C-terminal 6x His-tag, which could be removed with XhoI digestion to express only an N-terminal His-tag, which is in turn coded for by the plasmid. The plasmid His-tag could be removed with NcoI digestion to express only the C-terminal His-tag.

Before the proteins were expressed, the correct genes were assembled from the primary constructs in Table 1 and inserted into the expression vector. FastDigest (Thermo Scientific) restriction enzymes were used for all DNA-cutting purposes, and the cleaved DNA fragments were separated on a 1% TAE-agarose gel with a current of 90 V. The desired bands were cut from the gel and DNA fragments were purified using a GeneJET Gel Extraction Kit (Thermo Scientific). Fragments were ligated to the pBADHisBΔ-plasmid using T4 DNA ligase (New England Biolabs). The ligation product was transformed into heat competent TOP10 *Escherichia coli* cells, which were then incubated 1 h in 1 ml of 2xTY medium at 37 °C, rotating at 250 rpm on an orbital shaker. The cells were then spun down and plated on LB agar plates with 100 µg/ml ampicillin. After growing overnight, single colonies were picked and grown in 5 ml of 2xTY medium overnight at 37 °C, rotating at 250 rpm on an orbital shaker. Aliquots of the cultures were used to prepare stocks with 15% glycerol that were stored at -80 °C and then used to start production cultures. Plasmids were purified from the remaining precultures using GeneJET Plasmid Miniprep Kit (Thermo Scientific) and sent for Mix2Seq sequencing (Eurofins Genomics). If the plasmids had the correct sequence, the glycerol stocks could be used to start production cultures.

4.2. Protein work

4.2.1. Protein expression

Proteins were expressed in *E. coli* TOP10 heat-competent cells. Precultures of the bacteria were first grown overnight at 37 °C, rotating at 250 rpm on an orbital shaker in 5 ml 2xTY medium with 100 µg/ml ampicillin for selection. 2 ml of this preculture was used to inoculate 300 ml of 2xTY medium with 100 µg/ml ampicillin. The main cultures grew at 30 °C and 250 rpm until an optical density (OD_{600}) of approximately 0.7 was reached. At that point, 0.02% (w/v) of L-arabinose was added, which induces transcription of protein from the plasmids. For iron-containing proteins 0.1 mM of $Fe(SO_4)(NH_4)_2(SO_4)\cdot 6H_2O$ was also added at induction to ensure a sufficient iron source. Protein expression was done overnight at room temperature and 180 rpm.

4.2.2. Protein purification

Cells were harvested from the main cultures by a 20 min centrifugation at 4500 x g and resuspended in 25 ml wash buffer (Appendix 1), after which the cells were disrupted through sonication. Cell debris was centrifuged down at 43500 x g and the supernatant was extracted. The supernatant was incubated with TALON® Superflow™ resin (Clontech) at + 4 °C and the resin was collected and placed in a column. The resin contains cobalt ions, which have high affinity to the 6 x His-tags of the proteins. The resin was washed with wash buffer and protein was eluted with elution buffer (Appendix 1). During elution the imidazole in the elution buffer takes the place of the protein and binds to the cobalt ions in the resin, causing the protein to be washed out. The elution buffer was exchanged to storage buffer (Appendix 1) with PD-10 desalting columns (GE Healthcare) whereafter the protein was concentrated with 4 ml Amicon Ultra concentrators (Merck) and glycerol was added to a final concentration of 50%, after which the protein were stored at -20 °C.

4.2.3. Protein characterization

SDS-PAGE was used to verify that all the purified proteins had the correct molecular weight. SDS gives the protein a negative charge, after which an electrical current is used to separate proteins on a polyacrylamide gel according to their size. The molecular weights of the proteins were predicted based on their sequence using the ProtParam tool of the Expasy server (Wilkins et al., 1999). Molecular weights were checked against a marker of proteins with known molecular weights.

The concentration of all proteins except DoxA were measured using Bradford analysis, in which the absorbance of a protein sample with a colored reagent is measured and compared to the absorbance of a known standard of bovine serum albumin (Bradford, 1976). The ferredoxins and ferredoxin reductases were further characterized through UV/Vis-spectroscopy with a Multiskan™ GO (Thermo Scientific) plate reader to verify that they had absorption spectra characteristic of their respective protein classes.

DoxA was characterized using a CO-binding assay. This assay works for all CYP450 enzymes and can be used to determine both the concentration and the viability of the enzyme. Carbon monoxide binds irreversibly to reduced heme, and the CO-bound heme causes a characteristic absorption peak at 450 nm. Biologically inactive heme proteins will not be able to bind the CO and will have an absorbance peak at 420 nm. In order to do this, two parallel sample cuvettes of DoxA containing purified DoxA diluted 20-fold in 80 mM phosphate buffer pH 7.5 with 1 mM EDTA and 20% glycerol were prepared. An absorbance spectrum between 400 nm and 500 nm was measured for both samples as a baseline. In the sample cuvette, approximately 60 bubbles of CO-gas were slowly bubbled through the protein solution, saturating the sample with CO, which replaces O_2. The reducing agent sodium dithionite was added to both sample cuvettes and a second absorption measurement was recorded. The following formula was used to calculate the concentration of functional heme in the sample: $[(A450\text{-}A490)_{observed}\text{-}(A450\text{-}490)_{baseline}] \div 0.091 = \text{nmol per ml}$. The benefit of this assay is that it measures the presence of functional DoxA rather than total protein concentration, which is the case for Bradford analysis (Guengerich et al., 2009).

4.3. DoxA activity assay

The activity of DoxA was measured via the conversion of its substrates. DOD, DHD DNR were used as substrates for the assay. DoxA has a higher reported activity with DHD and DOD (Walczak et al., 1999), and therefore it was easier to do activity measurements with these substrates even though the aim of the research was production of DXR from DNR. The reaction is dependent on electrons derived from NADH or NADPH, and all reactions therefore contained a NAD(P)H constituting system made up of glucose, glucose dehydrogenase (GDH), $NAD(P)^+$ and NAD(P)H. These components create a cycle of NAD(P)H regeneration where GDH catalyzes the reduction of $NAD(P)^+$ with hydrogen from glucose, assuring that there is always an abundance of NAD(P)H in the reaction. In addition to this, reactions contained catalase and ascorbic acid to remove hydrogen peroxide and reactive oxygen species (ROS), phosphate buffer pH 7.5, DoxA, redox partners and substrate. The concentrations of the different components were determined after initial optimization reactions. Reactions were carried out in 0.25 ml volume at 30 °C and rotating at 250 rpm in the dark.

4.4. Analysis of reaction products

The reaction products were extracted with a 4:1 mixture of chloroform and methanol, which was then evaporated *in vacuo*. The dried product was resolubilized in methanol and analyzed by high-performance liquid chromatography (HPLC), using a SCL-10Avp/SpdM10Avp system with a diode array detector (Shimadzu), running samples through a C18 2.6-µm, 4.6 × 100-mm Kinetex column (Phenomenex) with a mobile phase gradient of 15-100% acetonitrile and 0.1 % formic acid. In HPLC, the organic compounds in methanol that are extracted from the reaction are bound to the stationary phase of the column. The compounds have different solubility in acetonitrile, so when the concentration in the mobile phase changes, the compounds will be eluted at different times. They will then show up as separate peaks in the UV/vis measurement.

An absorption peak around 470 nm is typical for anthracycline compounds and absorbance at that wavelength was therefore measured to characterize the reaction products (Perveen et al., 2018). These peaks were compared to standard peaks of DXR, DNR and DHD to detect substrate conversion.

5. Results

5.1. Protein purification and verification of molecular weights

Throughout the process of the thesis work, several different proteins were purified based on the original constructs in Table 1. The successfully purified proteins that were used in activity assays are outlined in Table 2. Concentrations were calculated using the Bradford method, apart from DoxA and DoxA_RhF, the concentrations of which were calculated with the CO-binding assay. The molecular weights of all proteins were verified by SDS-PAGE and found to be correct (data not shown). Production of X5 and any fusions containing X5 were unsuccessful, and no protein could be purified.

Table 2: All the purified proteins used in the DoxA activity assay

Protein	**Concentration**	**Protein**	**Concentration**
DoxA	42,56 µM	DoxA_RhF	34 µM
X4	394,9 µM	X4R4_PG	8 µM
PDX-PDR_PG	118,1 µM	YkuN	443 µM
R4	224,77 µM	YkuN-Fpr_P5	64 µM
R7	180,55 µM	PDR	117 µM
PDX	237,32 µM	X4R7_PG	41 µM

5.2. *S. peucetius* X4 is likely a Rieske-type [2Fe—2S] ferredoxin

The UV/vis spectrum of purified X4 shows peaks at 458 nm and 325 nm, as well as a shoulder at 560 nm. This aligns with reported spectra of oxidized Rieske type ferredoxins and differs from the spectrum of PDX, which is a non-Rieske ferredoxin (Figure 3). PDX exhibits peaks at approximately 330, 420 and 450 nm, which are to be expected in a non-Rieske [2Fe—2S] ferredoxin (Couture et al., 2001)(Fee et al., 1984). Both X4 and PDX are a visible rusty red color. Rieske-type ferredoxins differ from other ferredoxins in that the iron—sulfur cluster is not coordinated by four cysteine residues, but rather two cysteines and two histidines (Couture et al., 2001). Sequence analysis of X4 supports the hypothesis that it belongs to a different class of ferredoxins than PDX, as it shares high similarity with other Rieske-type ferredoxins and contains the conserved residues of that class (data not shown).

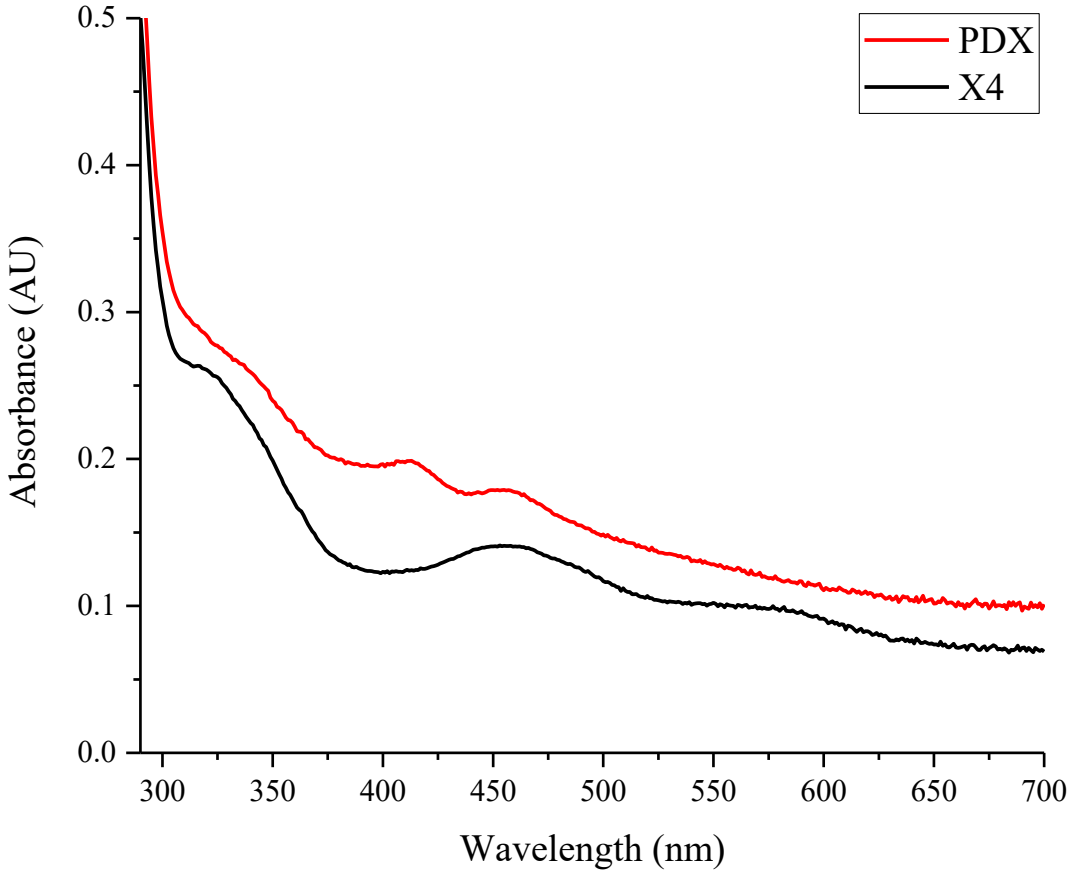

Figure 3: Absorption spectra of X4 (black) and PDX (red). Proteins were suspended in 20 mM phosphate buffer pH 7.5.

5.3. Putative *S. peucetius* ferredoxin reductases R4 and R7 exhibit expected spectroscopic properties

The UV-vis absorption measurements of R4 and R7 reveal a spectrum that resembles that of other *Streptomyces* ferredoxin reductases (Ramachandra et al., 1991)(Chun et al., 2007) and that of PDR (Figure 4). Major absorption peaks are visible at approximately 450 and 380 nm, with shoulders at approximately 480 nm, 365 nm and 430 nm. The proteins are also visibly bright yellow, which is characteristic for proteins containing flavin cofactors. It is likely that these proteins are in fact ferredoxin reductases.

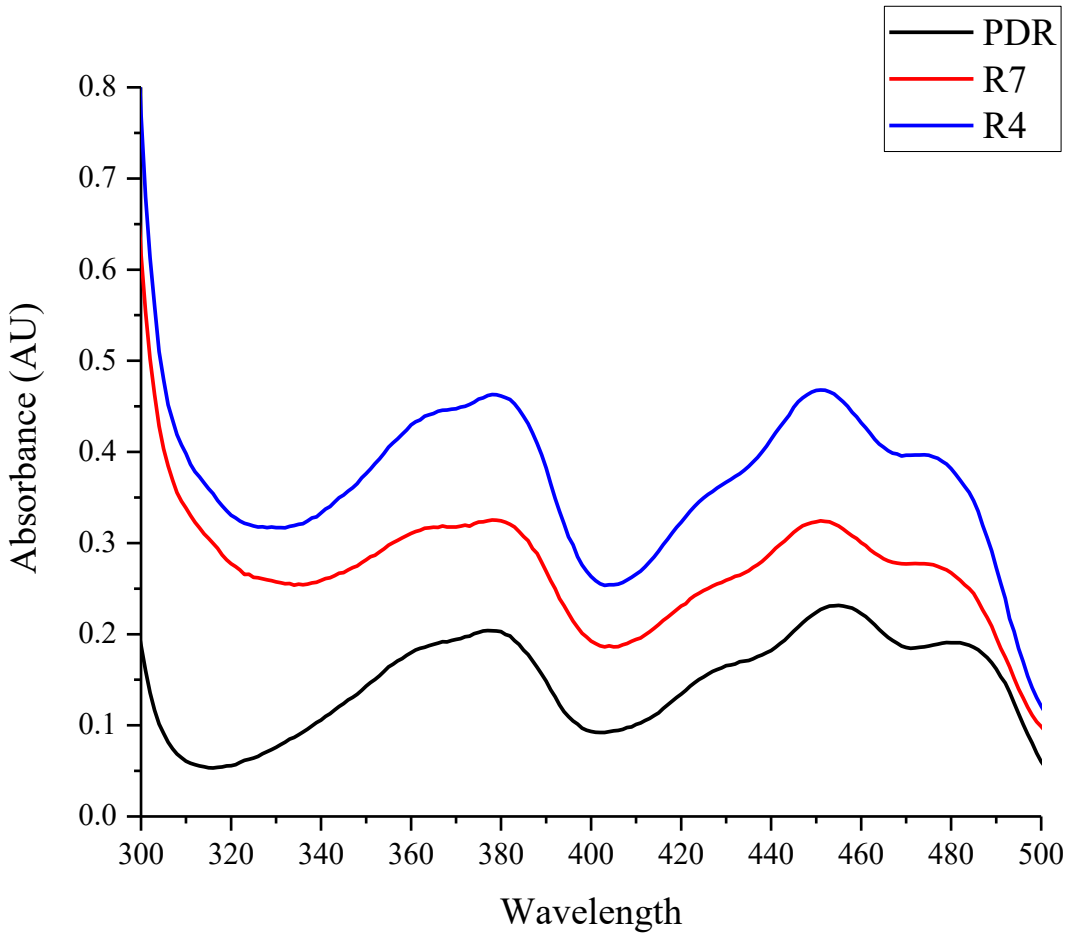

Figure 4: Absorption spectra of PDR (black), R7 (red) and R4 (blue).

5.4. Purified DoxA exhibits characteristic absorbance spectra upon reduction and CO-binding

The CO-assay of DoxA shows a clear difference in the absorbance at 450 and 420 nm in samples with and without CO-gas (Figure 5). Reduction with sodium dithionite has decreased the absorbance at 420 and increased the absorbance at 450 in both the control sample and the sample saturated with CO, but the change is greater in the sample with CO. Applying the formula $[(A450\text{-}A490)_{observed}\text{-}(A450\text{-}490)_{baseline}] \div 0.091 = \text{nmol per ml}$, yields a concentration of 2.082 µM functional heme in the 20-fold diluted sample. The concentration of the purified protein stock is therefore approximately 42 µM.

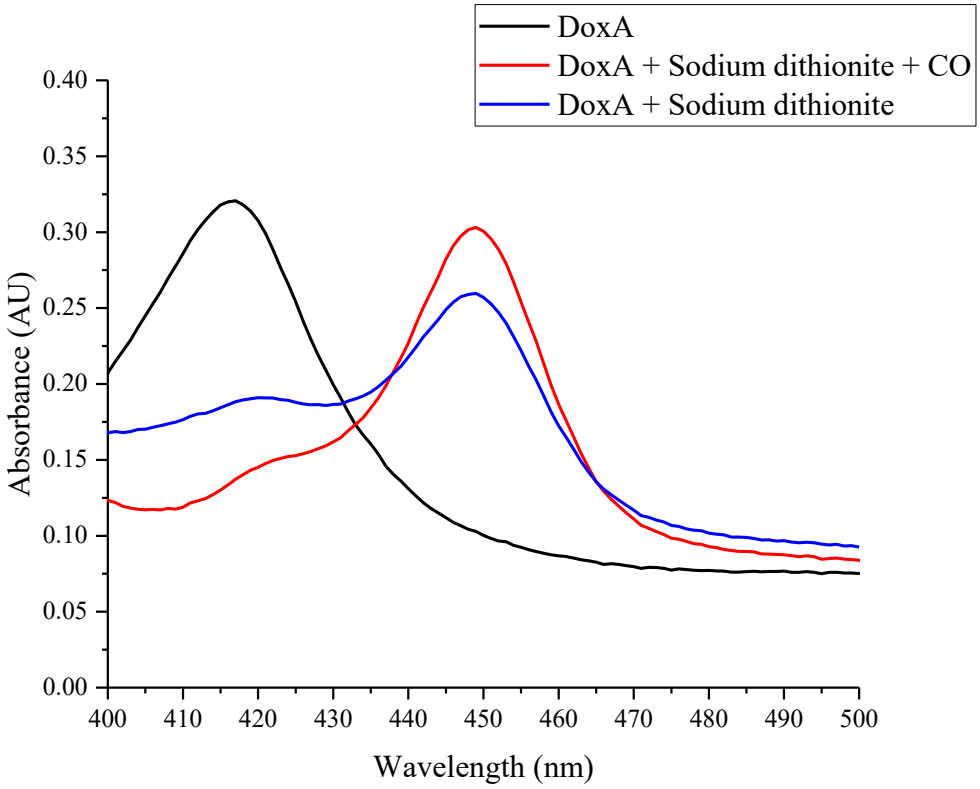

Figure 5: Absorption spectra of untreated DoxA versus those of DoxA treated with both CO and sodium dithionite or sodium dithionite only.

5.5. Poorly optimized reaction conditions of DoxA cause degradation and ineffective substrate conversion

The initial enzymatic assays of DoxA activity showed very poor activity regardless of the redox partners used. No conversion of DHD to DNR could be observed, and large unidentified peaks of degradation products were present in many reactions. Reactions with DOD as the substrate worked slightly better, but conversion was still poor and degradation products were present. 100 μM of substrate is often used in enzymatic assays with DoxA as described in literature. As seen in Figure 6, this substrate concentration did not work out for either DOD or DHD. Reducing the amount of substrate however, caused a decrease in degradation and increase in effectivity of conversion, with the best condition leading to complete conversion of DOD to DHD and further conversion to DNR. Figure 6 only shows reactions with YkuN-Fpr_P5 and the redox proteins from *P. putida*, since these were the pairs that displayed the best activity. Optimization was, however, also done with all other redox proteins. The optimization efforts also showed that longer reaction times did not help conversion, and neither did higher concentrations of DoxA or redox proteins.

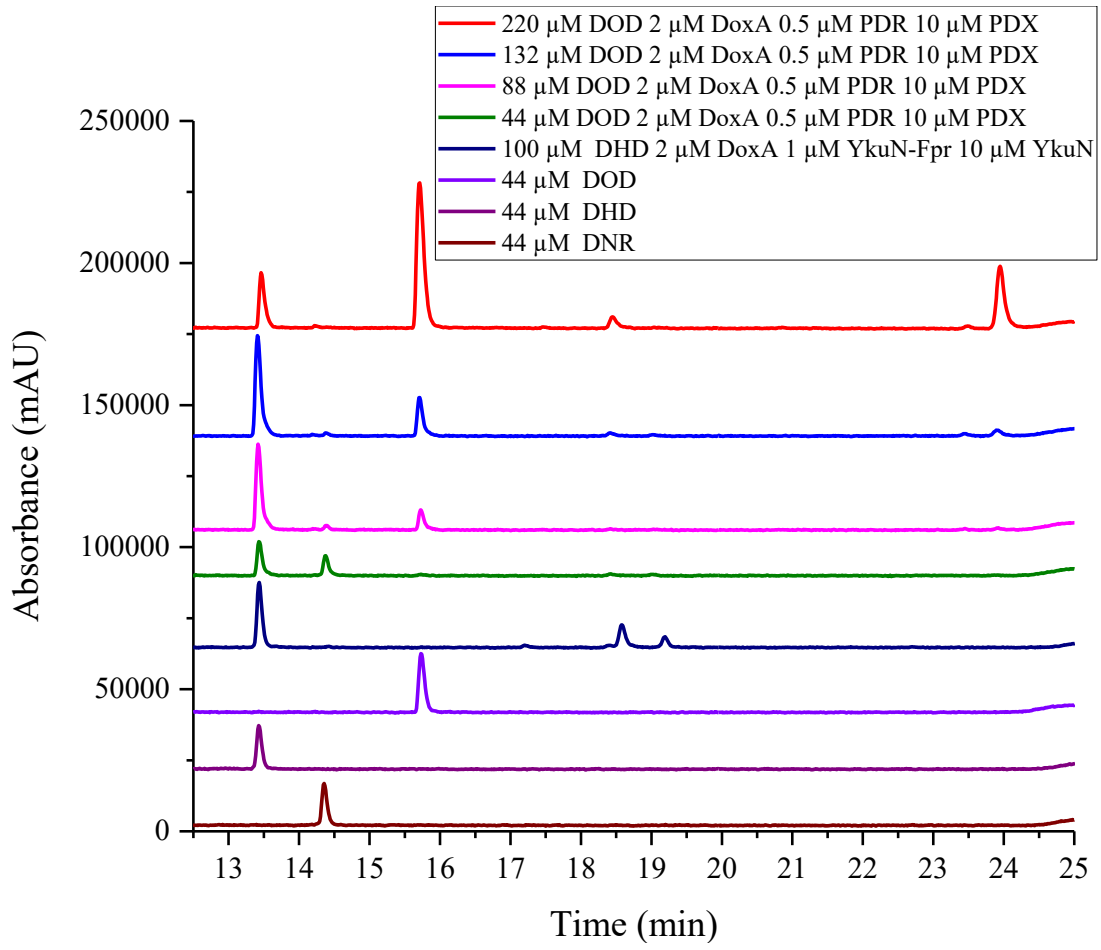

Figure 6: HPLC chromatogram of enzymatic reactions and standards of the associated compounds.

5.6. Purified *S. peucetius* redox proteins X4, R4 and R7 do not facilitate DoxA activity *in vitro*

Despite the success in optimizing the reaction conditions and seeing DoxA activity using the foreign redox proteins from *B. subtilis, E. coli* and *P. putida*, no activity could be observed when using the *S. peucetius*-derived X4, R4 and R7 in conditions that were optimal for other proteins. Figure 7 shows the complete lack of any conversion or even degradation of the substrate for all three substrates and all combinations of the three proteins.

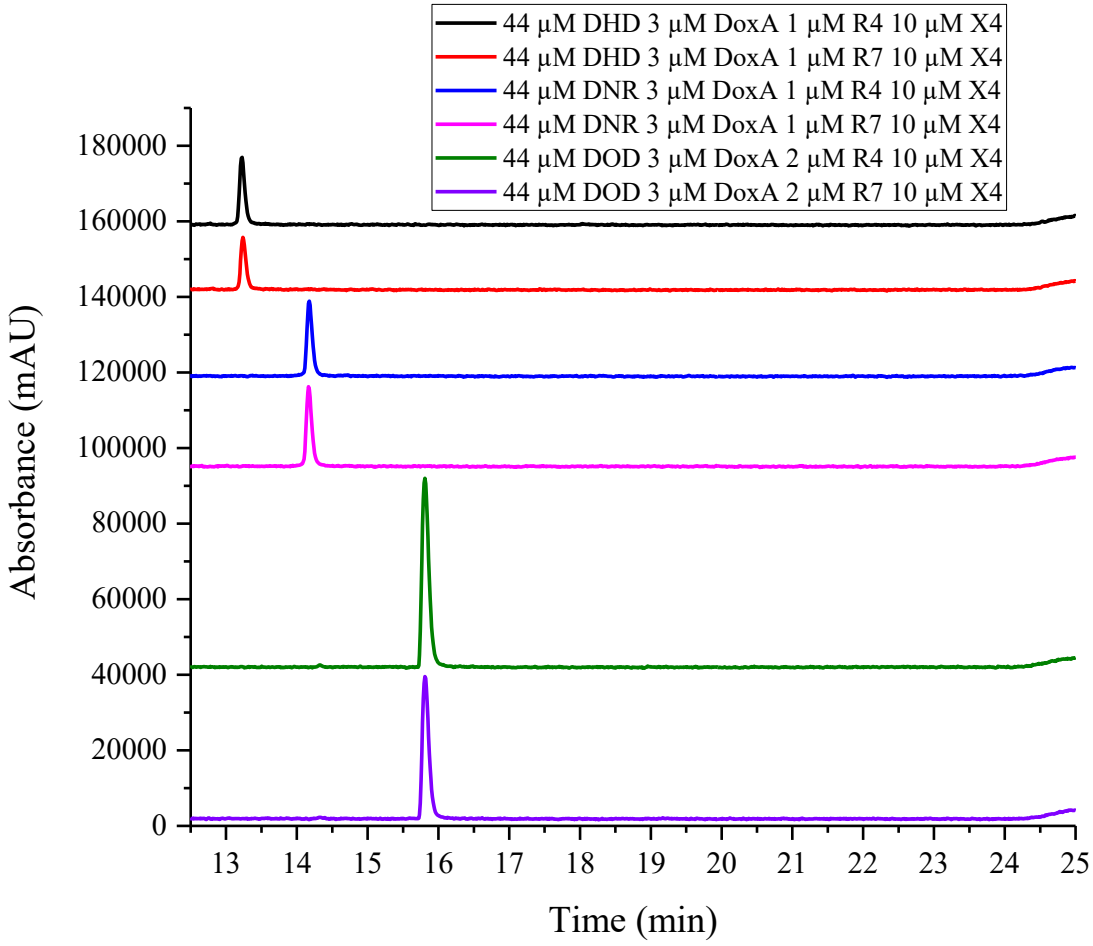

Figure 7: HPLC chromatogram of DoxA assays with S. peucetius redox enzymes.

The complete lack of activity persisted despite varied reaction conditions. Both NADH and NADPH were tried as electron sources, and the proteins were also coupled with PDR and PDX to see whether they were just not suited for each other. Furthermore, the fusions of X4 and R4 as well as X4 and R7, both with the PG-linker, were also tested, but showed an identical lack of activity. Figure 8 shows different attempts to see any activity with X4, R4 and R7. These reactions were carried out before the reaction had been fully optimized and therefore have a higher substrate concentration.

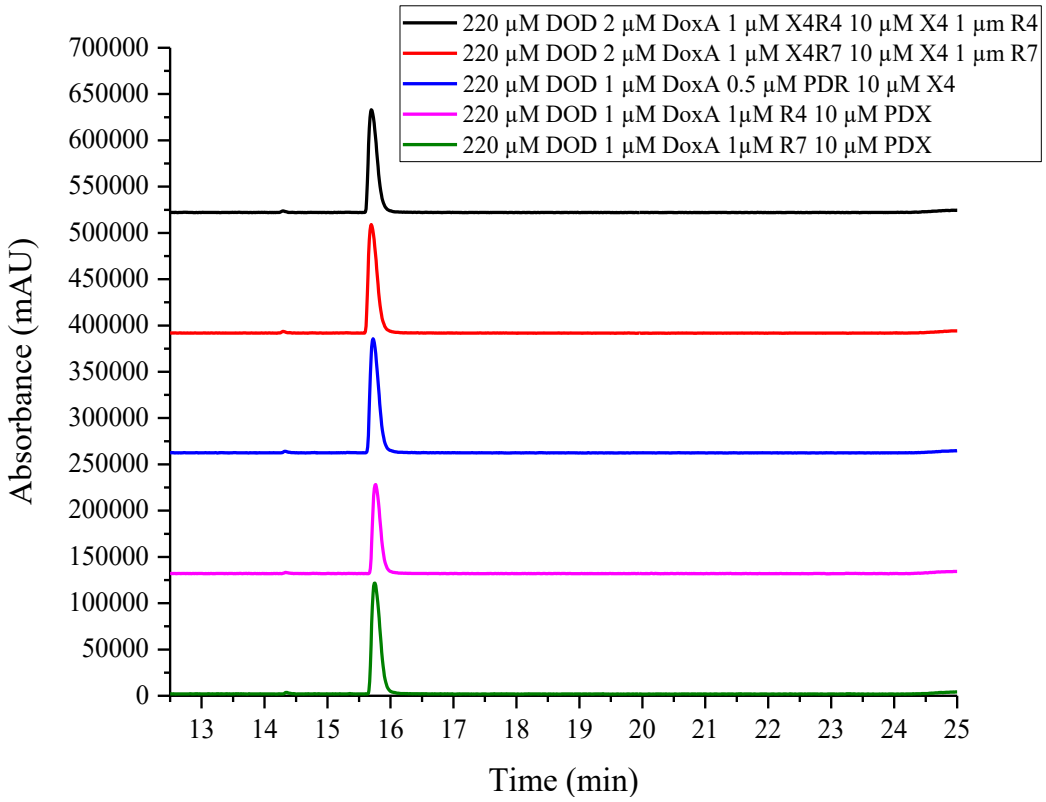

Figure 8: HPLC chromatogram of attempts to see any activity using S. peucetius derived redox proteins.

5.7. Foreign redox proteins can facilitate DoxA activity *in vitro*

While the redox proteins from *S. peucetius* that were predicted to be natural partners of DoxA did not facilitate substrate conversion at all, activity could still be reconstituted by using redox systems from other organisms. The proteins PDX and PDR caused the most effective substrate conversion and the YkuN-Fpr_P5 fusion protein together with an excess of YkuN was also highly effective. The DoxA_Rhf fusion caused some activity and the commonly used spinach redox proteins could also drive the reaction to some extent (Figure 9).

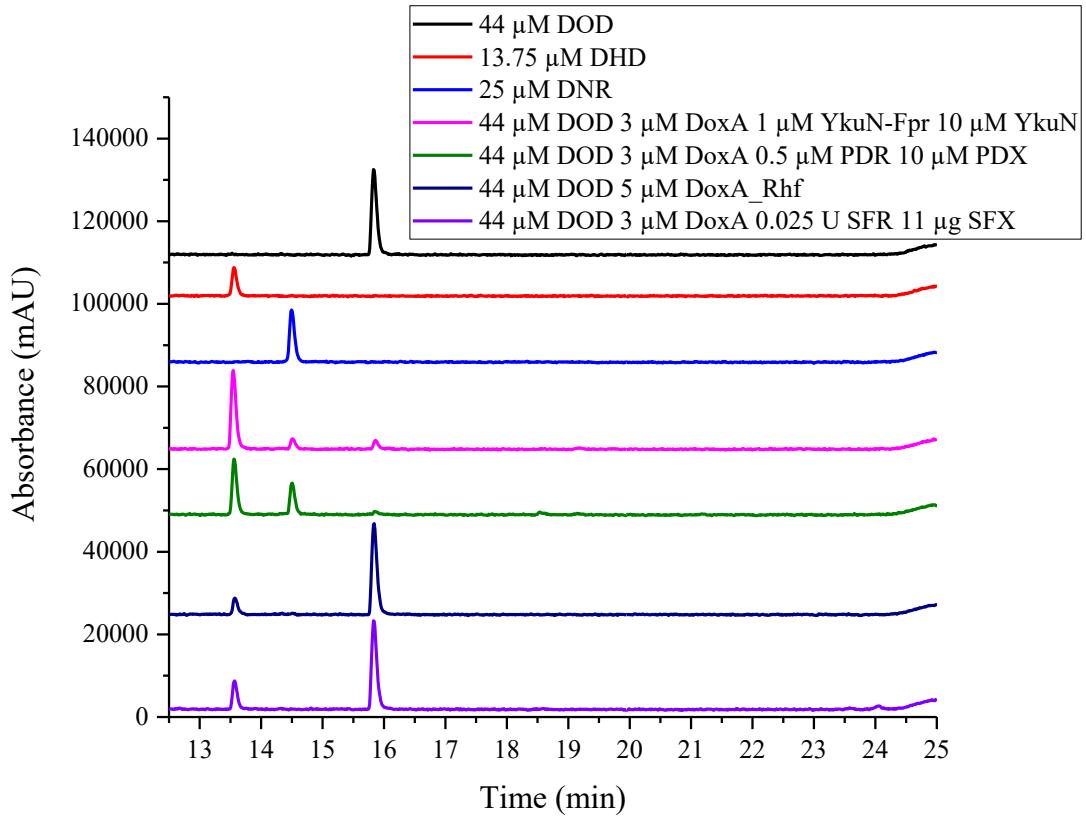

Figure 9: HPLC chromatogram of DoxA activity assays with DOD as substrate and associated standards.

When DHD was used as a substrate instead of DOD, conversion became visibly less efficient in all reactions. PDR with PDX could drive the reaction almost to complete conversion into DNR, while YkuN-Fpr_P5 with YkuN could only convert about half of the substrate. DoxA_Rhf and SFX with SFR both showed very similar activity, converting a small amount of DHD into DNR. The reactions with PDX and PDR, YkuN-Fpr_P5 and YkuN, and SFX with SFR all caused minor degradation peaks (Figure 10). It is unclear whether these peaks are caused by degradation of the substrate or of the product, or both.

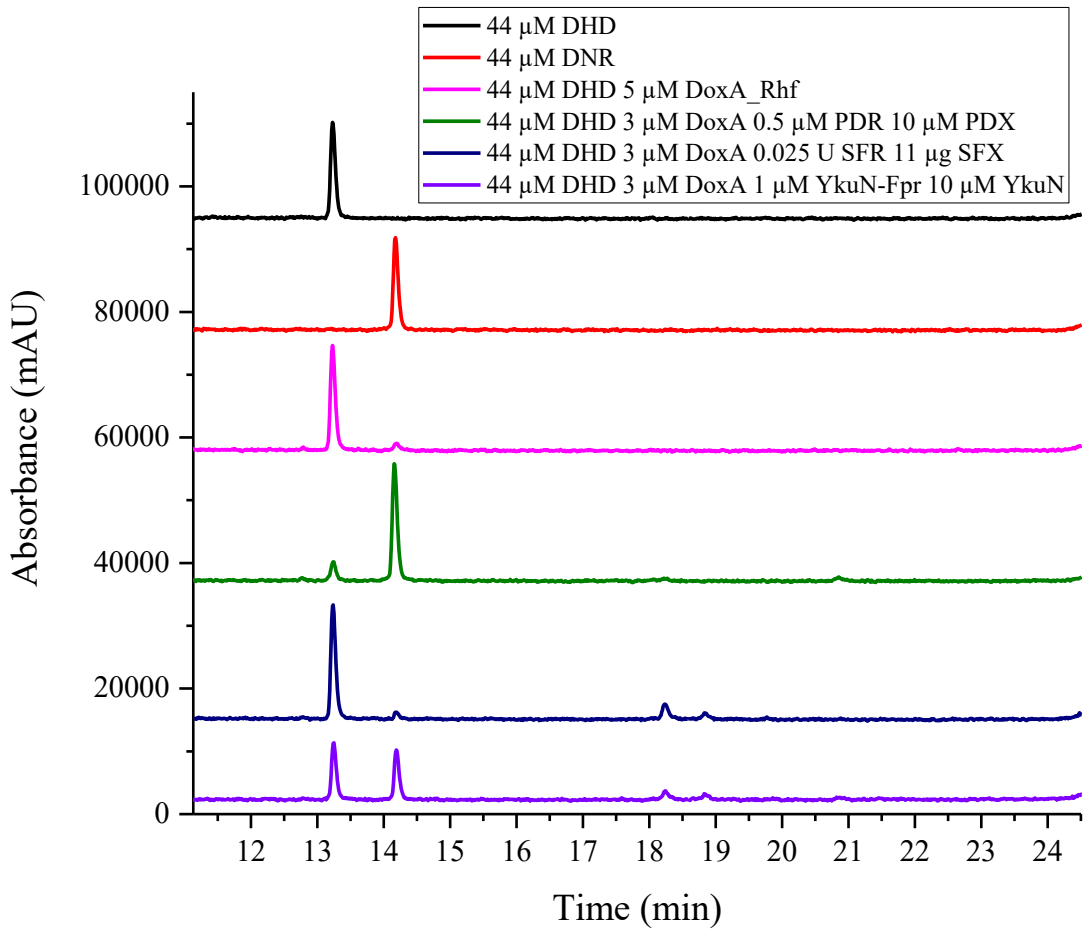

Figure 10: HPLC chromatogram of optimized enzymatic assays with DHD as substrate and associated standards.

In the final measurements, DNR was used as a substrate to see if any conversion into DXR could be shown. In these reactions, conversion was much worse, with PDR and PDX being the only pair that caused any conversion, and that conversion only resulted in a minimal peak of DXR (Figure 11).

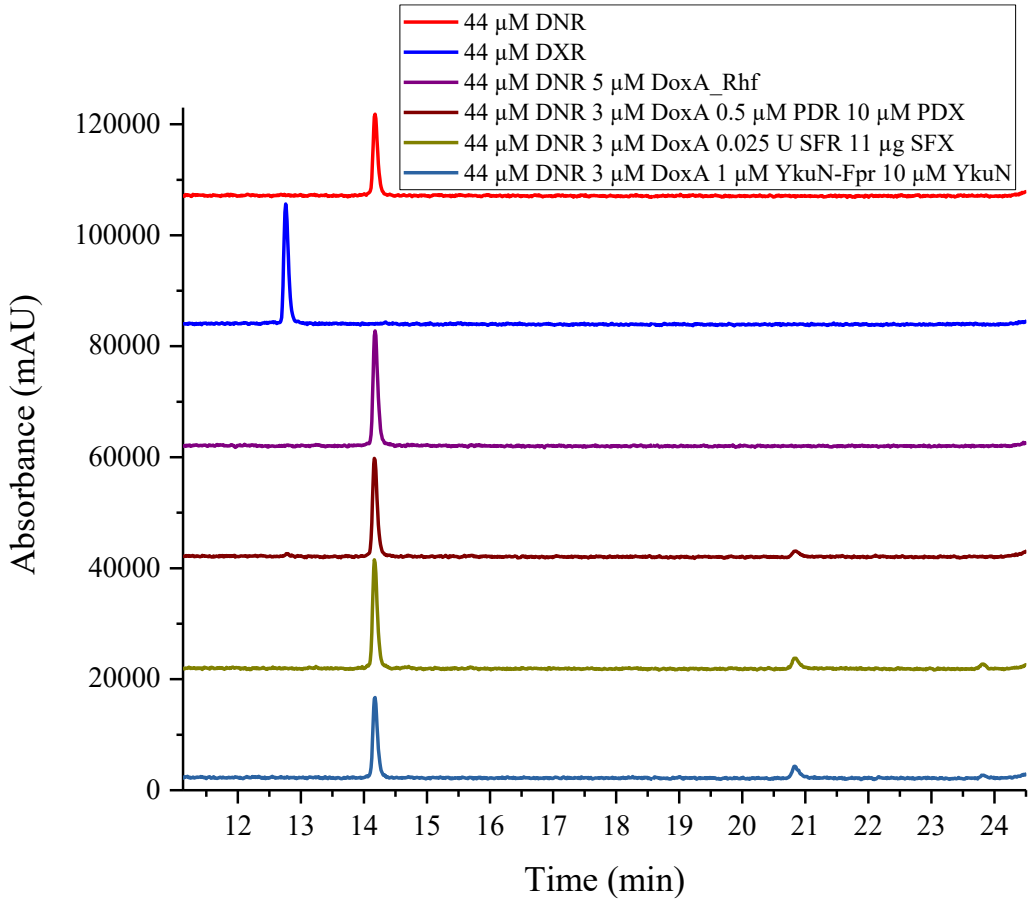

Figure 11: HPLC chromatogram of optimized enzymatic assays with DNR as substrate and associated standards.

5.8. Fusion of PDX and PDR with the flexible PG-linker impedes their ability to reduce DoxA

Since the YkuN-Fpr_P5 construct worked well, a similar construct was also made for PDX and PDR. The flexible PG-linker was used instead of the rigid P5 linker used in YkuN-Fpr_P5. The results show that the construct on its own does not facilitate any activity of DoxA when only the fused protein is used as a redox partner. If an excess of PDX is added, there is partial activity. This proves that at least the PDR part of the fusion protein is functional. The partial activity achieved with the fusion plus an excess of PDX is however lower than the activity of separate PDX and PDR (Figure 12).

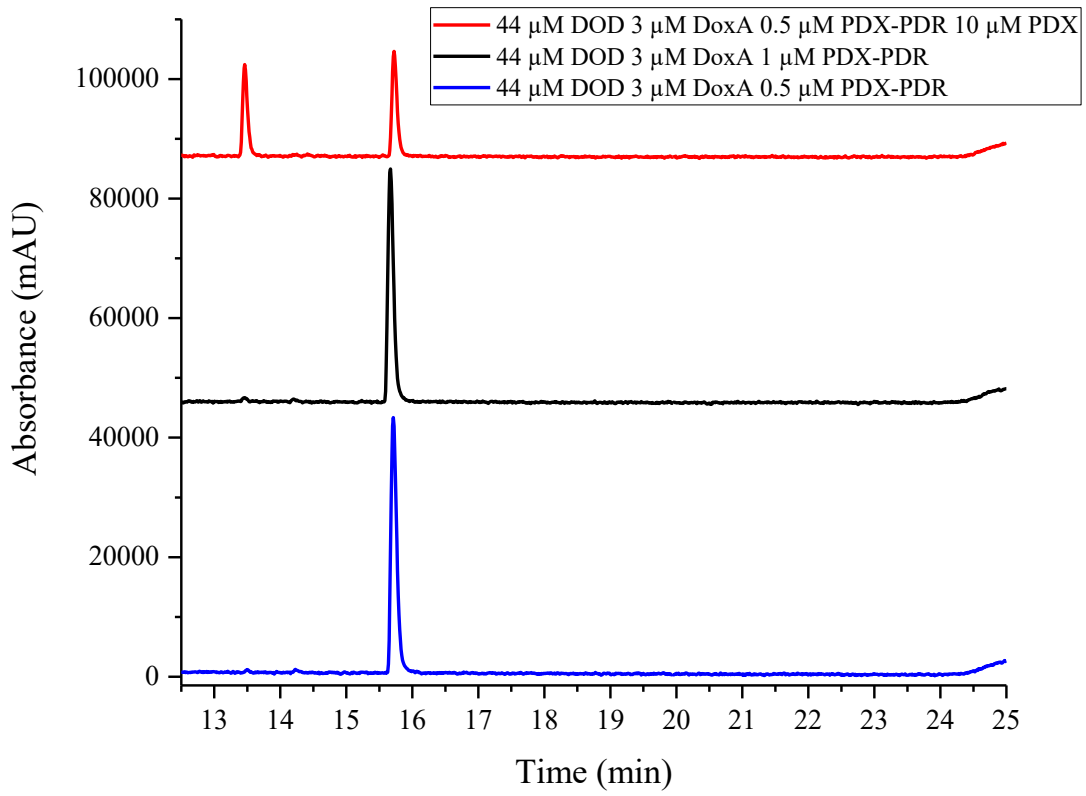

Figure 12: HPLC chromatogram of enzymatic activity assays with the PDR-PDX fusion and DOD as substrate.

6. Discussion

The experiments of this thesis were designed and carried out based on the assumption that differential regulation of redox proteins and related pathways could be a factor in determining the ability of a *S. peucetius* mutant strain to produce DXR. While the experiments have shown that redox proteins greatly affect the ability of DoxA to carry out its catalytic functions, no results have supported that the ferredoxins X4 and X5 as well as R4 and R7 have any influence on DoxA. There are many reasons as to why this could be the case, ranging from a poor choice of experimental method to the proteins not actually taking part in the natural biosynthesis of DXR. Although the effects of the *S. peucetius* derived enzymes could not be established, many other insights about redox engineering can be gleaned from the results of this thesis.

6.1. Foreign redox proteins and other unnatural redox systems are proficient DoxA reducers

The greatest successes of this thesis are the different unnatural redox systems that were constructed and shown to facilitate DoxA activity. The foreign systems that showed some activity were PDX and PDR, SFX and SFR, DoxA_RhF and YkuN-Fpr_P5. Of these, PDX and PDR, as well as SFX and SFR were previously known to reduce DoxA, and the proteins are commercially available. SFX and SFR seems to be the default redox system used by many when the interest of the study is just to see any activity without regard for efficiency. Examples of a DoxA_RhF fusion and YkuN-Fpr_P5 activating DoxA cannot be found in literature and they are not commercially available. PDX and PDR allowed for the highest activity and YkuN-Fpr_P5 with an excess of YkuN was also a good system. As for the DoxA_RhF fusion and the pair formed by SFX and SFR, they both showed low activity, not at all on the level of the better redox proteins. Out of the commercially available redox pairs, the PDX-PDR pair therefore seems to be the better of the two, and not the more commonly used SFR

and SFX. While these redox systems could activate DoxA and allow it to catalyze DOD and DHD, they did not seem to cause any differences in the production profiles of DoxA or cause it to prefer one substrate over another.

This knowledge is useful in the future when research on the topic of DoxA and redox proteins is explored further. The systems established herein can be used as positive controls to make sure that purified DoxA is active and able to function. They could also be used in assays that study the effects of different mutations of DoxA, like inactivation of key residues or even mutations aiming to improve DoxA activity. It would also be interesting to see what effect PDR and PDX, or the YkuN-Fpr_P5 fusion have when inserted into DNR- or DXR- producing strains of *S. peucetius*. Even though they did not make DoxA synthesize DXR *in vitro*, they might still have some effect in an *in vivo* system with its more complex environment and possibilities for further interactions.

6.2. Conversion of DNR to DXR *in vitro* could not be achieved

Despite numerous attempts to optimize the enzymatic assay for the various redox proteins used, no condition that facilitated significant conversion of DNR to DXR could be found. Conditions that allowed for almost complete conversion of DOD to DHD and DHD to DNR were only able to cause a minimal peak of DXR, visible in Figure 11. This sharp drop in DoxA activity seems surprising at first but makes sense when examined in a greater context. The literary sources that describe *in vitro* DoxA assays all report very low conversion of DNR to DXR. In Rimal et al., 2015, for example, a conversion of only 7.5% is achieved, and DoxA is deemed active based on this. Walczak et al., 1999 showed that conversion of DNR to DXR is significantly less efficient and slower than the other two reactions carried out by DoxA, based on assays carried out with cell lysate instead of purified proteins. Walczak et al., 1999 also reported that DXR acts as a powerful inhibitor of DoxA. The data obtained in this thesis also suggest that DOD, DHD and DNR are also inhibitory at high concentrations, since DoxA activity decreased drastically at higher substrate

concentrations. The reason for the degradation that took place at higher substrate concentrations might be found in the interactions between the redox proteins and anthracyclines. Previous studies have shown that ferredoxin reductases can directly reduce anthracyclines, daunorubicin among them, under certain circumstances, causing degradation of the molecule (Fisher, Abdella & McLane, 1985). Optimization results showed that the degradation was dependent on ferredoxin reductases, and it is possible that adding an excess of substrate created conditions where the substrate, instead of DoxA, was more susceptible to reduction.

S. peucetius strains are dependent on self-resistance genes in order to not succumb to the anthracyclines they produce. The most important of these genes are *drrA, drrB* and *drrC*. *drrC* encodes a protein that repairs DNA damaged by DXR (Lomovskaya et al., 1996), whereas *drrA* and *drrB* encode transporter proteins that make the cell permeable to DXR (Kaur, 1997). These transport proteins might be the key to why conversion of DNR to DXR is possible *in vivo* but seems to be more difficult *in vitro*. When DXR is produced in the cell it is soon transported out and the ratio of DNR to DXR is such that DXR keeps being produced. In the assay system used in this thesis, the DXR that is initially produced has nowhere to go. In addition, the surrounding conditions of the *in vitro* environment might lack something that is found in the cell and is beneficial for DXR synthesis. It is possible that the tiny DXR peak visible in Figure 11 is enough to inhibit the further conversion of DNR in the conditions present in the enzymatic assay.

6.3. *S. peucetius* derived redox proteins X4, X5, R4 and R7 did not facilitate any DoxA activity

X4 and X5 were chosen as likely candidates for interacting with DoxA and enabling conversion of DNR to DXR based on RNA-sequencing data done on DXR overproducing strains of *S. peucetius*. No ferredoxin reductases stood out especially in the transcriptomics data, and therefore R4 and R7 were chosen to go with these based on homology to a ferredoxin reductase from *Pseudomonas* sp. that is known to

interact with a homolog of X4 (Senda et al., 2007). Despite the sound reasoning behind the choice of *S. peucetius* redox proteins to test and the numerous different optimization attempts of the reaction, no DoxA activity whatsoever could be observed when using X4, R4 and R7 for electron transport. Fusions of X4 to R4 or R7 did not fare any better, and neither did pairings of *S. peucetius* redox proteins and *P. putida* redox proteins. The spectroscopic verification of the proteins shows that they have the characteristic features and cofactors that are to be expected. This implies that they are functional. Production of X5 was not successful and therefore it could not be tested in the activity assay.

Initially, the activity assay results seemed to indicate that X4 is not the correct protein and that it cannot interact with DoxA, and that perhaps X5 would have worked if it could only have been produced. However, DoxA does not seem to be very selective about what redox protein it gets its electrons from, as evidenced by the fact that DoxA showed slight activity with SFX and SFR derived from spinach, which is a species that belongs to a different kingdom of life than *S. peucetius*. It seems unlikely that DoxA would show no activity at all with a native ferredoxin. The issue was further complicated when other endeavors within this research project obtained results from *in vivo* studies of DXR producing strains that showed that overexpression of X4 drastically increased the production of DXR. The engineered strains showed almost complete conversion of DNR to DXR. There is therefore reason to believe that X4 is in fact a redox protein that can facilitate DXR overproduction, just as predicted. Hence, the reason behind the failure of the *in vitro* experiments must lie elsewhere.

The most likely solution seems to be that X4 is not compatible with any of the ferredoxin reductases it was used alongside. Even though it would be able to reduce DoxA, this cannot happen due to X4 not being reduced first. R4 and R7 were selected based on information about homologous proteins, but this is not a guarantee that they will work with X4. Single amino acid mutations can be enough to interfere with the ability of two proteins to interact with each other. *S. peucetius* has five more ferredoxin reductase candidates that could work with X4 and among these one should be able to find a protein that can reduce DoxA together with X4.

There are of course other possible reasons why the *in vitro* assay did not work with X4, R4 and R7. Iron—sulfur proteins are known to be sensitive to aerobic conditions,

[4Fe—4S] proteins more so than [2Fe—2S] proteins. X5 is predicted to be [4Fe—4S] or [3Fe—4S], which would explain why no protein could be produced under the aerobic conditions used in protein purification. While X4 should be more stable with regards to its iron—sulfur cluster, there is still a chance that the cluster is somehow broken, despite it looking spectrophotometrically sound. Further validation using some technique that can monitor the flow of electrons between the proteins could be used to make sure that the cluster is functional. This could be done with stopped-flow spectrophotometry or electron paramagnetic resonance spectroscopy.

6.4. The validity of fusion proteins in redox engineering

The results of the fusion proteins used in the assays are both poor and inconclusive. The only one that worked properly was YkuN-Fpr_P5, and that one also required an excess of YkuN to achieve maximal activity. PDX-PDR_PG showed no activity on its own but did gain some activity when an excess of PDX was added. This indicates that transfer from PDR to PDX within the fusion did not occur, but that at least PDR was functional and could transfer electrons to the free PDX. These two fusion proteins differ in the type of linker that was used – PG is a flexible linker due to the interspersed glycine residues whereas the high proline content of the P5 linker makes it rigid. In this case it is not possible to say that the type of linker is what caused one fusion to work and not the other, since the P5 linker was not tested with PDX and PDR due to time constraints. The P5 linker is directly copied from Bakkes et al., 2017 and it is the optimized linker for YkuN-Fpr, but the assumption cannot be made that it would work with PDR and PDX just because it works well with the other two proteins. If it is indeed the linker that is the problem in PDR-PDX_PG, then it is possible that the flexibility of the linker is somehow allowing the two proteins to maintain some position relative to each other that does not allow electron transfer. In that case, it would make sense that an optimized rigid linker that keeps the two proteins at the appropriate distance and orientation to each other would work better, as is the case with YkuN-Fpr_P5. Further, it has been shown that PDR can dimerize in solution (Kuznetsov et al., 2005), which might be important for its function and fusing the

protein with another protein might hinder dimerization. Other types of complex formation, for example with the P450, might also be hindered by the protein fusion.

Unfortunately, the *S. pecuetius* derived redox enzymes did not exhibit any activity at all and therefore the impact of fusions on those proteins could not be judged. If they would have worked, more time would probably have been spent trying out the different linkers and optimizing the reactions with fusion proteins. This would have given further insights into the feasibility of fusing redox proteins and given more insight about the proteins themselves. The results from the two different fusions presented now are hardly enough for any definitive conclusions.

With all of the above in mind, one can still speculate about the role of fusion proteins in general. Bakkes et al., 2017 managed to build a well-working construct, but the fact remains that even in the best scenarios, the fused protein could only achieve 96% of the activity of free proteins, and this was with the addition of 10-fold excess YkuN. In other words, they could not show a reaction where fusion made electron transport more efficient than separate proteins. It also remains a fact that ferredoxins or flavodoxins are often the limiting components in the electron transport chain and that a 1:1 ratio of reductase to ferredoxin/flavodoxin is often going to be suboptimal.

The above does not mean that no good protein fusions of this type can be made for any reaction, but it does speak to the difficulty of making an efficient and "correct" fusion that brings proteins together without disrupting any of their other functions. It seems reasonable to assume that in order to build good fusions, more advanced technologies and modelling tools than what have been used in this case are needed. If it would be possible to accurately model the interactions and complexes of a specific electron transport pathway, there would be a much better starting point to tailor a fusion of proteins for that pathway. One could take into account the exact distances and orientations needed between the proteins and cofactors and possibly also fuse more than two proteins if dimers are needed. These kinds of fusions would not be applicable outside a specific pathway, but might make a single reaction more efficient.

What then is the role of fusion redox proteins as they are presented herein? In the case of YkuN-Fpr_P5, the fusion protein solves solubility issues of Fpr, which tends to be a difficult protein to produce and purify (Bakkes et al., 2017). This can also be the case for other proteins that have solubility issues. If a system can be made that works

to a sufficient degree without needing excess ferredoxin or flavodoxin, it would reduce the number of different proteins that need to be purified for a reaction. This might in some cases be a better solution even if the fusion is not quite as effective as the separate proteins. In conclusion, fusion proteins do have a role to play in redox engineering, but in the experiments of this thesis they did not turn out to be useful. There is still potential to be found in fused redox proteins and technological advances will probably make them more useful in the future.

7. Conclusions

The aim of this thesis work was to find ways to control and manipulate the activity of the CYP450 monooxygenase DoxA via the redox systems its function relies on. The problem was approached from several different angles and several different systems were planned out and constructed. Fusion proteins of FDXs and FDRs were built, as well as self-sufficient DoxA fusions. Redox proteins from other organisms were purified with hopes that they might give new functionality to DoxA. The assay that measured the activity of DoxA was proven to work after a long time spent optimizing reaction conditions and results showed that DoxA activity could be constituted to varying degrees using several redox systems. The most relevant redox proteins to study, the FDXs and FDRs from natural DXR producers, did not constitute even the slightest activity. No redox systems could drive the reaction all the way to DXR synthesis, which might be an indicator of the *in vitro* system having poor validity in replicating the events that take place during biosynthesis of DXR in *in vivo* systems. Despite all this, there are still valuable results obtained from the experiments. New systems were proven to be able to reduce DoxA, e.g. the fusion protein YkuN-Fpr_P5, which is the first example of a flavodoxin reducing DoxA. The self-sufficient DoxA_RhF was slightly active and could probably still be improved. PDX and PDR were shown to work better than commonly used spinach redox proteins.

In hindsight it is clear that the thesis work might have benefitted from a more focused approach to redox engineering. Instead of focusing on a combination of foreign proteins, self-sufficient fusion and fused redox proteins, it might have been wiser to go all in on a single aspect, and then if necessary, also look into the others. The experiments regarding fusions of redox proteins suffered especially as a result of this. PDX-PDR_PG and YkuN-Fpr_P5 were the only fusion proteins that showed any activity, but since they have different linkers it is hard to make any kind of comparison between the two. More time or more focus would have benefitted any aspect of the research. As it now stands, the project only managed to scratch the surface of redox engineering.

Based on what has been shown in this thesis and in other literature regarding redox engineering, it is safe to say that redox engineering is and will continue to be a possible

avenue to explore when it comes to engineering CYP450-enzymes. It does however seem like the main application of redox engineering is to ensure a more effective supply of electrons to the CYP450, since that is a relatively simple thing to achieve using systems that are known to work well in many cases. This approach can be used to create simpler systems that do not require as many components or operate faster. In cases like DoxA, where it is known that the enzyme has many natural substrates and there is some indication that its substrate selectivity might be linked to the expression, it is reasonable to try to use redox engineering as a way to alter the production profile. However, if there is no such clue to be found, it is very difficult to find a redox system that would cause the desired change in production.

For this project, the next logical step is to focus on the *S. peucetius*-derived redox enzymes and try to find the physiological redox proteins involved in DXR biosynthesis. Since the *in vivo* data shows that an abundance of X4 seems to cause DXR overproduction, it is of utmost interest to identify the correct reductase for that protein. The safest way to make sure that the correct protein is found is to produce and purify all the putative ferredoxin reductases from DXR-producing strains and do DoxA activity assaying of them together with X4, following the method established in this thesis.

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9. Swedish summary – Svensk sammanfattning

Redoxproteinteknik som verktyg för förbättring av DoxA-aktivitet

9.1. Introduktion

Cancer är i dagens samhälle en av de vanligast förekommande dödsorsakerna, och i och med den ökande kemiska bördan samt befolkningens åldrande ökar incidensen alltjämt (Bray et al., 2018). Effektiv behandling av cancer är ett mål som genomsyrar en mycket stor del av den forskningen som genomförs inom biovetenskaperna. I denna kamp har man ofta vänt sig till naturen för att hitta medel att bekämpa sjukdomen med. Ett av dessa medel är doxorubicin (DXR), en förening som produceras av vissa stammar av bakterien *Streptomyces peucetius* subsp. *caesius* (Arcamone et al., 1969). DXR är ett av de mest använda cancerläkemedlen och används i behandlingen av många olika cancertyper. Den biosyntetiska produktionen av DXR i stammar av *S. peucetius* subsp. *caesius* är väldigt ineffektiv, då låga halter av DXR produceras i förhållande till prekursorn daunorubicin (DNR) och de två föreningarna är utmanande att separera från varandra. Därför produceras DXR i dagens läge utgående från bioproducerat DNR, vilket är en kostsam och arbetsdryg process (Weiss, 1992). I denna pro gradu undersöks möjligheter att förbättra DXR-produktionen i bakterierna genom så kallad redoxproteinteknik av proteinet DoxA, som står för katalysen av de sista reaktionerna i biosyntesen av DXR.

DXR hör till klassen antracykliner. Antracycliner är polyketider som består av en fyraringad aglykonstruktur och en sockermolekyl som är bunden till C-7 på aglykonen. DXR produceras i en komplicerad biosyntetisk process där många enzymer katalyserar reaktioner som slutligen leder till föreningen 13-deoxydaunorubicin (Dickens, Priestley & Strohl, 1997). De tre sista hydroxyliseringsreaktionerna, från DOD till 13-dihydrodaunorubicin (DHD), DHD till DNR och slutligen DNR till DXR, katalyseras av enzymet DoxA (Walczak et al., 1999). DoxA tillhör enzymklassen cytokrom P450 (CYP450), vilket är en grupp enzymer som karakteriseras av sin hemgrupp, som de använder sig av för att katalysera

diverse reaktioner. För att kunna genomföra sin katalytiska funktion är CYP450-enzymerna beroende av molekylärt syre och elektroner, som de får via elektrontransportproteiner. CYP450-enzymer kan katalysera ett stort antal olika sorters reaktioner, och många av dem är svåra och dyra att efterlikna på syntetisk väg. Enzymerna är ett mycket intressant forskningsmål inom proteinteknik, eftersom de potentiellt kan användas som alternativ för dyra kemiska processer på industriell skala (Girvan, Munro, 2016).

Proteinteknik med CYP450-enzymer kan göras på många sätt, t.ex. genom att ändra på proteinets sekvens för att uppnå stabilare eller effektivare protein. Ett sätt som inte kräver modifieringar av själva enzymet är redoxproteinteknik. Med redoxproteinteknik menas att man byter ut eller på annat sätt modifierar det system som förser CYP450-enzymet med elektroner. Detta kan orsaka förändringar i CYP450-enzymets effektivitet eller till och med i dess substratselektivitet (Zhang et al., 2018). I naturen förekommer ett flertal olika sorters transportsystem, varav det vanligaste är det så kallade klass I-systemet. Det består av två delar: ett ferredoxinreduktas och ett ferredoxin. Ferredoxinreduktaset transporterar via sin flavindinukleotidkofaktor (FAD) elektroner från elektronkällorna NADH eller NADPH till ett ferredoxin. Ferredoxinet har ett Fe—S-kluster som kan ta emot elektroner och sedan reducera hemgruppen som finns i CYP450. Andra redoxsystem finns också, t.ex. flavodoxiner som har en flavinmononukleotidkofaktor (FMN) i stället för Fe—S-klustret (Girvan, Munro, 2016). Vissa enzymer, t.ex. P450RhF är självförsörjande och behöver bara en elektronkälla för att fungera. P450RhF är en fusion av ett CYP450-enzym och en RhF-domän som innehåller både FMN och ett [2Fe—2S]-liknande kluster (Nodate, Kubota & Misawa, 2006). DoxA fungerar antagligen med hjälp av ett Klass I-system, men de associerade redoxproteinerna har inte identifierats. Dock har man identifierat flera förmodade ferredoxiner och ferredoxinreduktas i *S. peucetius* genom, och högst antagligen finns bland dessa det proteinpar som förser DoxA med elektroner (Rimal et al., 2015). Transkriptomanalyser som gjorts som en del av det förberedande arbetet för detta projekt tyder på att det kan finnas ett samband mellan ökad DXR-produktion och uppreglering av ferredoxinerna X4 och X5. Homologidata tyder på att dessa kunde fungera med ferredoxinreduktaserna R4 och R7.

I pro gradu-projektet används tre typer av redoxproteinteknik: redoxproteiner från främmande arter, fusioner av redoxproteiner och fusion av DoxA till RhF för att göra ett självförsörjande enzym. Alla dessa tillvägagångssätt kan potentiellt påverka DoxA:s aktivitet eller substratselektivitet.

9.2. Målsättningar

Projektet undersöker olika redoxsystems inverkan på DoxA in vitro. Detta görs dels med onaturliga redoxsystem, d.v.s fusioner och proteiner från främmande arter, och dels med hur några redoxproteiner som härstammar från *S. peucetius* och tros kunna vara DoxA:s naturliga redoxproteiner. Eftersom proteinerna från *S. peucetius* inte har studerats tidigare är det även viktigt att karakterisera dem för att säkerställa att de faktiskt är den sortens proteiner som de förmodas vara. Projektets målsättningar kan sammanfattas på följande sätt:

- Att ta reda på hur olika redoxsystem påverkar DoxA:s katalytiska aktivitet och substratselektivitet.
- Att karakterisera de förmodade redoxproteinerna X4 och X5 samt R4 och R7 från *S. peucetius.*
- Att få insikter som kan användas för att producera DXR in vivo.

9.3. Material och metoder

Allt arbete inom projektet gjordes in vitro, vilket innebar omfattande kloningsarbete samt porteinproduktion och –rening. Alla proteiner som renades hade sitt ursprung i 7 syntetiska gener (Tabell 1).

Tabell 1: Syntetiska gener som användes som grund för kloning och proteinproduktion

Gen	Länk	His-tag
YkuN-Fpr	P5	N/C
X4R4	PG	N/C
X5R7	P5	N/C
PDX	-	N
PDR	-	N
DoxA	-	N
RhF	Nativ	-

Dessa gener kunde med hjälp av molekylärbiologiska metoder klippas och ligeras för att skapa gener för olika fusioner eller separata proteiner. För fusionsproteinerna fanns två olika alternativ för länksekvenser: den flexibla PG-länken och den rigida P5-länken. För fusionsproteinerna kunde jag även välja mellan att uttrycka dem med C-terminal eller N-terminal His-tag.

Proteinerna producerades i vätskekulturer av TOP10 *Escherichia coli*-bakterier. Proteinerna renades från cellerna genom affinitetskromatografi av cellysatet. För affinitetskromatografin användes en solid fas innehållande koboltjoner som binder till det producerade proteinets His-tag. De renade proteinerna koncentrerades och kunde därefter förvaras i -20°C. Proteinerna karakteriserades med hjälp av diverse spektrofotometriska metoder. Ferredoxin och ferredoxinreduktas från *Spinacia oleracea* (spenat) producerades inte själv, utan köptes från kommersiella källor.

DoxA-aktivitet mättes med hjälp av ett enzymatiskt test där DoxA blandades med substrat, redoxproteiner, elektronkälla och ett regenerationssystem för elektronkällan. Alla tre substrat, d.v.s. DOD, DHD och DNR testades. Reaktionsblandningen inkuberades 1 timme i 30°C, varefter överblivet substrat och reaktionsprodukter extraherades från vattenfasen med hjälp av en blandning av kloroform och metanol i förhållandet 4:1. De extraherade ämnena analyserades med hjälp av högupplösande vätskekromatografi (HPLC). HPLC separerar föreningar baserat på deras

hydrofobicitet och mäter deras absorbans, vilket ger upphov till ett kromatogram där de olika föreningarna syns som absorbanstoppar med olika retentionstid. Dessa kromatogram analyserades för att kunna dra slutsatser om hur effektivt de olika redoxsystemen kunde aktivera DoxA.

9.4. Resultat

Produktionen av alla proteiner förutom X5 och fusioner som innehöll X5 var lyckad. Alla renade proteiner påvisade korrekta spektrofotometriska egenskaper och de kan därmed antas ha de rätta kofaktorerna och vara aktiva. Aktivitetsmätningarna visade att man med många redoxsystem kunde uppnå konversion från DOD till DHD, och till viss del från DHD till DNR. Inga redoxsystem kunde dock få DoxA att hydroxylera DNR till DXR. Det redoxsystem som fungerade bäst var de separata proteinerna putidaredoxin (PDX) och putidaredoxinreduktas (PDR) från *Pseudomonas putida.* Detta par gav upphov till komplett konversion av DOD till DHD och nästan komplett konversion från DHD till DNR. Fusionensproteinet YkuN-Fpr_P5, med ett överskott av separat YkuN gav bra resultat med DOD som substrat, men kunde bara konvertera runt hälften av DHD till DNR. Fusionen DoxA_Rhf påvisade liten aktivitet med både DOD och DHD, men aktiviteten var betydligt sämre än med YkuN-Fpr_P5 samt PDR och PDX. Redoxproteinerna från spenat presterade på samma nivå som DoxA_Rhf. Fusionen PDX-PDR_PG visade ingen aktivitet utan extra PDX. Med ett tillsatt överskott av PDX var konversionen ändå sämre än YkuN-Fpr_P5 med ett överskott av YkuN.

Redoxproteinerna från *S. peucetius*, d.v.s X4, R4 och R7, kunde inte ge upphov till någon som helst DoxA-aktivitet. Alla kombinationer av ovanstående proteiner testades, både som separata proteiner och fusionsproteiner. Många olika proteinkoncentrationer och reaktionsförhållanden testades också, men DoxA-aktivitet kunde ändå inte konstateras.

9.5. Diskussion och avslutning

Resultaten visade att DoxA inte verkar vara speciellt selektivt när det kommer till vilket system det får sina elektroner ifrån. DoxA kunde till och med reduceras av proteiner från ett helt annat taxonomiskt rike, d.v.s. spenatproteinerna. Jag kunde även visa att ett flavodoxin, YkuN, kunde reducera DoxA och att fusionen av YkuN och Fpr fungerade väl i detta sammanhang. I och med att reducering av DoxA inte verkar vara så svårt att åstadkomma ter det sig märkligt att redoxproteinerna från *S. peucetius* inte kunde orsaka någon som helst DoxA-aktivitet. Dessutom har in vivo-tester inom en annan del av detta projekt visat att överexpression av X4 i DXR-producerande stammar leder till en signifikant ökning i mängden DXR som produceras i förhållande till DNR. Detta tyder på att X4 borde kunna reducera DoxA. En möjlig orsak till att jag inte kunde se någon aktivitet i reaktioner med X4 och DoxA är att ferredoxinreduktasen R4 och R7 inte kan interagera med X4. Tester visade att de andra ferredoxinerna och ferredoxinreduktaserna inte fungerade när man blandade ”fel” proteiner, vilket tyder på att interaktioner fellan ferredoxiner och ferredoxinreduktaser kan vara ganska specifika.

Även om konversionen av DOD till DHD och DHD till DNR bevisar att de olika redoxsystemen reducerade DoxA, kunde ingen konversion från DNR till DXR påvisas. I och med att DXR är den viktigaste produkten i sammanhanget och den som är svårast att producera är detta något av en besvikelse. Det är dock inte helt oväntat, eftersom DoxA:s affinitet till DNR är mångfalt lägre än till DOD och DHD. Andra studier där man har undersökt DoxA-aktivitet in vitro har även lidit av detta problem (Rimal et al., 2015) och det verkar som om in vitro-metoder inte lämpar sig för DXR-produktion. En möjlig förklaring till detta är avsaknaden av de DXR-transportsystem som finns i in vivo-system. Eftersom DXR är en inhibitor av DoxA måste föreningen transporteras bort från cellen med hjälp av specialiserade transportproteiner för att biosyntesen ska kunna fortgå (Lomovskaya et al., 1996). Dessa mekanismer finns inte i in vitro-system och det är möjligt att små mängder av DXR produceras, vilket sedan inhiberar fortsatt produktion. För att vidare undersöka interaktioner mellan X4 och DoxA kan de resterande ferredoxinreduktaserna från *S. peucetius* produceras och testas på samma sätt som de andra proteinerna i denna avhandling.

Insikterna från detta projekt tillsammans med resultaten från in vivo-arbetet som skett vid sidan om kan i fortsättningen användas för att vidare utveckla och optimera biosyntesen av DXR. Resultaten stöder också de grundprinciper som redoxproteinteknik baserar sig på, och jag tror att framtidens tekniska och vetenskapliga framsteg kommer att göra redoxproteintekniken till ett livskraftigt alternativ när det gäller proteinteknik som riktar in sig mot CYP450-enzymer.

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Appendix 1: Recipes

Luria-Bertani medium

10 g tryptone

10 g NaCl

5 g yeast extract

1 l tap water

2 x TY medium

16 g tryptone

10 g yeast extract

5 g NaCl

1 l tap water

Wash buffer for protein purification

5 mM imidazole

10% glycerol

50 mM Tris pH 7,5

300 mM NaCl

Elution buffer for protein purification

250 mM imidazole

10% glycerol

50 mM Tris pH 7,5

300 mM NaCl

Storage buffer for purified proteins

10% glycerol

100 mM Tris pH 7,5

600 mM NaCl

Phosphate buffer 100 mM

20,214 g Na_2HPO_4 heptahydrate

3,394 g Na_2HPO_4 monohydrate

1 l distilled water