DIRECTED EVOLUTION OF A CYTOCHROME P450 ENZYME TO INCREASE PEROXIDATION ACTIVITY

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Bioengineering

by
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December 2019
İZMİR

ACKNOWLEDGMENTS

First and foremost, I would like to thank my supervisor Asst. Prof. Nur Başak SÜRMELİ for her help, care and support throughout this project and my graduate education. It would not be possible to come this far without her continuedly and ingeniously guidance.

I would like to thank The Scientific and Technological Research Council of Turkey (TUBITAK) (116Z380) for financially supporting this project. Additionally, my sincere thanks to İzmir Institute of Technology, Biology and Bioengineering Applications and Research Center (BIYOMER) for their contributions in this study.

Also, I would like to thank Prof. Dr. Hüseyin Çağlar KARAKAYA and Assoc. Prof. Dr. Gülistan MEŞE ÖZÇİVİCİ for opening me their laboratory during this study.

More importantly, I would like to express my gratitude to Sürmeli Laboratory members; Yaprak ASLANTAŞ, Ekin KESTEVUR DOĞRU, Gülce GÜRALP, Fatmanur BOSTAN, Tuğçe SAKALLI, Dilara TİLKİOĞLU and Alper ŞAHİN for their precious friendship and generous help.

I am thankful to my office mates; Eyüp BİLGİ, Gülten KURU, Yiğit Ege ÇÖMLEKÇİ, Öykü SARIGİL and Gamze DOĞAN, Göklem ÜNER, Ünver KURT for their support in my difficult times. Also, I would like to thank Süleyman Can ÖZTÜRK, Elvan ÖZTÜRK, and İbrahim ÇELİK who encouraged me to start my master's degree.

Finally, my deepest appreciations and love goes to my family members, who helped in their special way. The most special thanks go to my beloved fiancée Fulya BAŞ, there are no words that can explain her support and care for me.

ABSTRACT

DIRECTED EVOLUTION OF A CYTOCHROME P450 ENZYME TO INCREASE PEROXIDATION ACTIVITY

Directed evolution, mimicking the natural selection, is a powerful tool to create novel enzymes. Evolution of natural enzymes to achieve desired properties are performed in iterative rounds of random mutagenesis followed by a screening/selection method. Enzyme activity can be enhanced with substituting the active site amino acids in the enzyme. CYP119, a member of cytochrome P450 protein family, is a thermophilic enzyme extracted from *Sulfolobus acidocaldarius* that exhibits monooxygenase, peroxidase and oxidoreductase activity. These properties give CYP119 a potential to be used in production of fine chemicals and pharmaceuticals. Herein, two different mutant libraries of CYP119, containing substituted amino acids at Thr213-Thr214 and Val151-Phe153 positions, constructed via combinatorial active site saturation test (CAST), and screened for improved peroxidation activity. Additionally, fluorescence based Amplex Red peroxidation activity assay using hydrogen peroxide as cofactor of CYP119 was optimized. Screening of mutant libraries resulted four improved CYP119 mutant enzymes from Thr213-Thr214 mutant library. Val151-Phe153 mutant library did not yield any improved peroxidation activity mutants which indicated amino acid substitutions at 151-153 positions do not have any effect on peroxidation activity of CYP119. Furthermore, effect of substituted amino acids at predetermined positions were analyzed. Substrate, Amplex Red, makes single or double hydrogen bond when molecular docking was performed on improved mutant enzymes also distance of nitrogen atom in Amplex Red to heme iron is closer than wild type CYP119 in improved mutant enzymes. Thus, increasing the peroxidation activity of mutant CYP119 enzymes.

ÖZET

PEROKSİDASYON AKTİVİTESİNİ ARTIRMAK İÇİN SİTOKROM P450 ENZİMİNİN YÖNLENDİRİLMİŞ EVRİMİ

Yönlendirilmiş evrim, doğal seçilimden esinlenilmiş ve yeni enzimler tasarlamak için kullanılan etkili bir yöntemdir. Doğada bulunan enzimlere, istenilen özellikleri eklemek ya da geliştirmek için, ötelemeli rastgele mutasyon ve devamında tarama/seçme metodu uygulanır. Enzim aktiviteleri, enzimin aktif kısmında bulunan amino asitlerin değiştirilmesi ile yükseltilebilir. Sitokrom P450 protein ailesinin üyesi olan CYP119, *Sulfolobus acidocaldarius*'tan izole edilmiştir ve termofilik bir enzimdir. Buna ek olarak CYP119 enzimi monooksijenaz, peroksidaz ve oksidoredüktaz aktivitesi göstermektedir. Bu özellikleri ile CYP119 enzimi saf kimyasalların ve farmasötiklerin üretiminde kullanılmak üzere potansiyel taşımaktadır. Bu çalışmada, Thr213.-Thr214 ve Val151-Phe153 pozisyonlarında değişiklik yapılmış iki farklı CYP119 mutant kütüphanesi artırılmış peroksidaz aktivitesi için taranmıştır. Buna ek olarak, hidrojen peroksiti CYP119'un kofaktörü olarak kullanan, floresan tabanlı Amplex Red testi optimize edilmiştir. Mutant kütüphanelerinin taranması, Thr213-Thr214 mutant kütüphanesinden dört adet peroksidasyon aktivitesi geliştirilmiş mutanın açığa çıkarılmasını sağlamıştır. Val151-Phe153 kütüphanesinden peroksidasyon aktivitesi geliştirilmiş mutant bulunamamıştır ve 151-153 pozisyonlarındanki amino asit değişikliklerinin aktiviteye herhangi bir etkisi olmadığı gözlemlenmiştir. Ek olarak, önceden belirlenmiş pozisyonlardaki amino asitlerin değiştirilmesinin etkileri de incelenmiştir. Moleküler doking uygulandığında, Amplex Red substratı, özellikle geliştirilmiş mutant enzimler ile tekli veya ikili hidrojen bağı yapmaktadır. Bununla birlikte, geliştirilmiş mutant enzimlerde, Amplex Red'de bulunan azot atomunun heme grubunda bulunan demir atomuna, yabanıl tipe göre, daha yakın olduğu gözlemlenmiştir. Böylece, seçilen mutant CYP119 enzimler geliştirilmiş peroksidasyon aktivitesine sahip olmuştur.

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CHAPTER 1

INTRODUCTION

1.1. Protein Engineering

Protein engineering aims to create novel proteins by modifying a protein sequence. Sequence of a protein is changed to achieve desired protein features such as, change in substrate specificity, increased stability towards temperature, organic solvents, extreme pH, selectively target other proteins or to design biomaterials [1, 2].

Enzymes are proteins that catalyze chemical reactions by regulating the rate of reaction. Enzymes have diverse application areas, including chemical, pharmaceutical, fuel, food, or agriculture industries. Naturally available enzymes usually do not fit to industrial applications and increasing demand of important enzymes in industry brought the need of novel biocatalysts with desired characteristics. Introduction of recombinant DNA technology has accelerated the development of novel biocatalysts. Engineering of enzymes is a part of protein engineering which introduces the predetermined changes in the amino acid sequence to achieve novel enzymes with desired properties. Protein engineering approaches are focused on improving functional properties of enzymes, such as activity, stability, substrate specificity, selectivity and solubility[2].

Engineering a protein generally involves three steps; selecting the changes in sequence of protein, applying these changes to sequence, and evaluation of protein variants for improved characteristics. Changes in protein sequence are determined by using protein engineering strategies; rational design or directed evolution. Modifications to sequences are applied via selected mutagenesis method, targeted mutagenesis or whole gene mutagenesis, followed by a selection or screening method to detect improved variants[3].

1.2. Protein Engineering Approaches

Protein engineering generally follows two approaches, rational design and directed (molecular) evolution, to create novel improved enzymes. A graphical explanation of rational design and directed evolution is given in Figure 1.1.Semi-rational evolution approach, combination of directed evolution and rational design, is also emerging in the last decade[4].

1.2.1. Rational Design

Rational design approach uses computational tools to design proteins with enhanced or novel functionalities. In order to rationally design a protein, knowledge of structure, function and mechanism of the protein is essential. Experimentally verified protein structural information [via X-ray crystallography or Nuclear Magnetic Resonance (NMR)] is limited to approximately 2% of proteins, for the rest prediction of protein structure is the only possible way[5]. Rational design is a powerful tool to redesign proteins with desired properties but despite remarkable examples there are many cases that have failed. These failures may occur from an incomplete understanding of protein mechanism and examining only primary amino acid sequence to introduce changes in protein sequence. Studying only primary amino acid sequence generally leaves out the structural properties of proteins. Such studies lead to a changes in strictly conserved amino acid residues resulting in an inactive conformation of protein that does not grant the desired enzyme properties[6]. For the rational design approach initially, crystal structure of protein is required, this is generally obtained by X-ray diffraction or NMR. After examination of protein structure and analysis of literature, possible mutation sites are determined. This knowledge is used to introduce changes in protein sequence. Site directed mutagenesis is widely used in rational design approach. With the help of computational tools, possible outcomes of amino acid changes at selected sites are observed and mutants with the desired properties are selected. Mutant genes are created according to determined changes and cloned into expression vector. Expression vector containing mutant gene, is

transformed into suitable host, such as bacteria, yeast, fungi, insect or mammalian cells. The most common host is bacteria especially *E. coli.* Mutant proteins are expressed, purified and subjected to preferred selection/screening method. Proteins with mutations are compared to wild type (WT) proteins to determine changes or improvements in the desired properties.

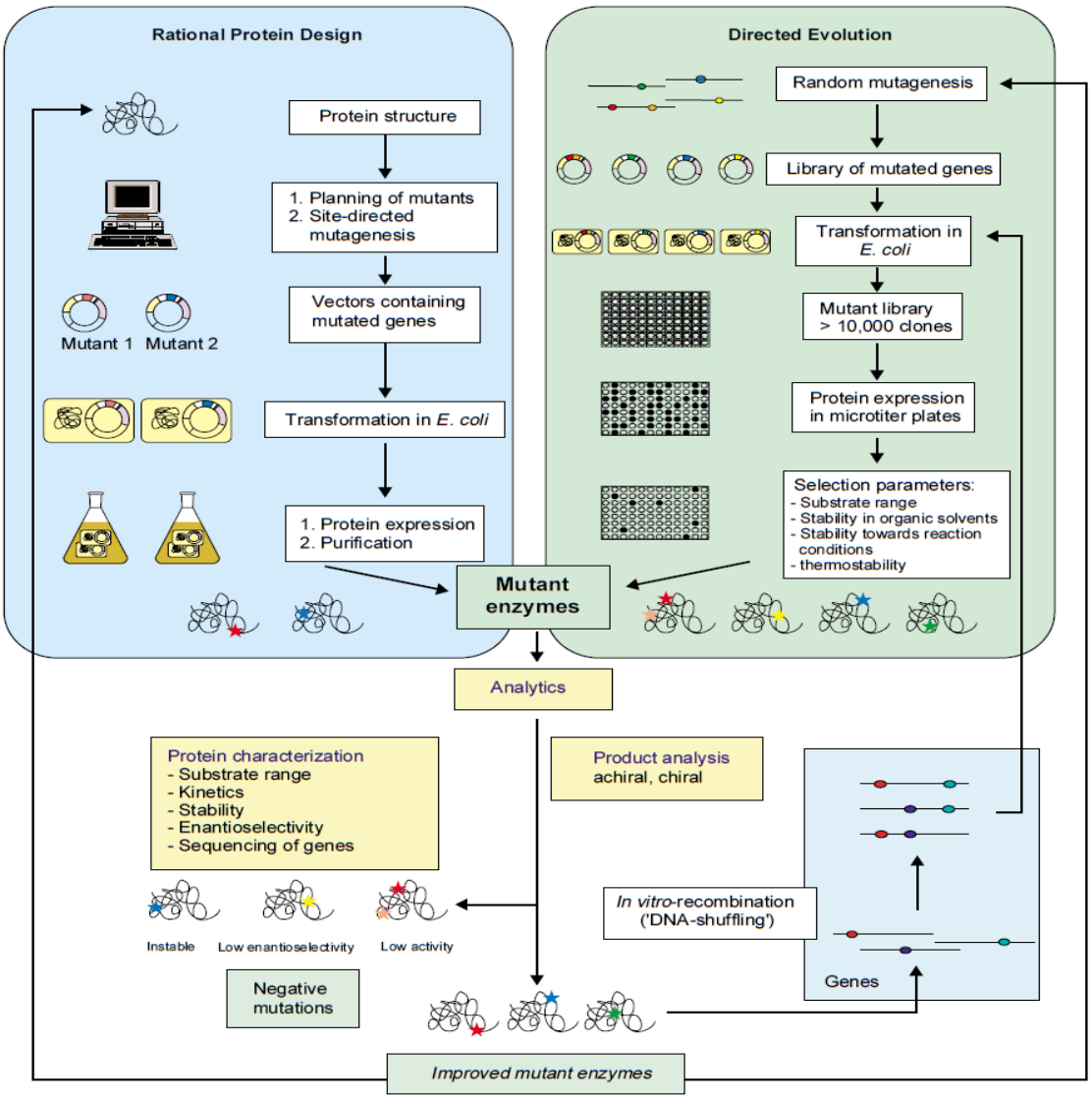


Figure 1.1. Graphical illustration of rational design, directed evolution and *in vitro* recombination followed by screening and characterization steps (Bornscheuer et al., 2001).

1.2.2. Directed Evolution

Directed evolution, inspired by natural selection, is an effective strategy to create proteins with desired properties. It is based on creation of diversity followed by a selection/screening method[7]. Diversity is created by introducing mutations into whole gene or particularly targeted sites. Random mutagenesis methods target the whole gene and among the techniques used are error-prone polymerase chain reaction (epPCR), chemical mutagenesis, UV irradiation or mutator strains. The most common random mutagenesis method is epPCR that has modified reagents or modified reaction conditions. A thermostable DNA polymerase that lacks proofreading ability, varying concentrations of dNTPs or Mn^{2+} instead of Mg^{2+} are among modified reagents. Despite having a mutational bias, commercially available DNA polymerases that are engineered to balance transitions and transversions can eliminate biases. In addition to reagents, also reaction conditions may vary, changing the annealing temperature or number of thermocycles contributes to random mutations[8]. Chemical mutagens, such as formic acid, ethyleneimine, nitrous acid, hydroxylamine, are also used to create mutations in DNA sequence. Chemicals can change the specificity of hydrogen bonding in nucleotides that lead to changes in base pairings[9]. Additionally, base analogues are used to introduce mutations. A base analogue is a chemical compound that has a similar structure to one of the bases of DNA. Base analogues have different base pairing characteristics than DNA bases, they replace the bases and cause mutations. The most common and widely used base analogue is 5-bromouracil which is a thymine analogue and pairs with adenine[9]. Mutator strains and UV irradiation are used to introduce mutations to whole gene. These methods offer skipping low-yield ligation steps, however there is a possibility of deleterious mutations on the host genome including vital genes such as, resistance genes[10]. Gene shuffling is based on homologous recombination of DNA *in vitro*. Stemmer and co-workers introduced DNA-shuffling method which involves digesting a gene with DNase I into small fragments and reassembling them into a full-length gene with repeated cycles of annealing with the help of PCR. Genes that were fragmented may either be a wild type homologs of a gene family or mutants of a particular gene that was created by using other mutagenesis method[11].

Site directed mutagenesis (SDM) is a practical tool to create mutant proteins, it is used to create specific, targeted changes in protein encoding gene. SDM sites can be determined from the primary protein sequence, as well as from structural data when it is readily available. While applying targeted mutagenesis methods, primers, that have appropriate degenerate codons (Table 1.1) according to desired requirements, are designed to cause mutations either on a single site or multiple sites on DNA template. Saturation mutagenesis methods check effects of all 20 common amino acids at the predetermined sites. Site saturation mutagenesis method is generally used to introduce mutations to a targeted site in order to generate smaller libraries[12].

Reetz and co-workers introduced a new approach Combinatorial Active Site Saturation Test (CAST) in 2005 [13]. CAST method substitutes amino acid pairs in the active site of the protein that are spatially close to each other as shown in Figure 1.2. Having two amino acids that are spatially close may grant synergistic conformational effects sourced from side-chain orientations. This is an unpredictable occurrence that cannot be achieved by single-site saturation mutagenesis. CAST method allows for systematic creation of small focused mutant libraries and reduce the screening effort. Screening of these libraries leads to positive hits therefore detection of significant regions around the active site of a protein.

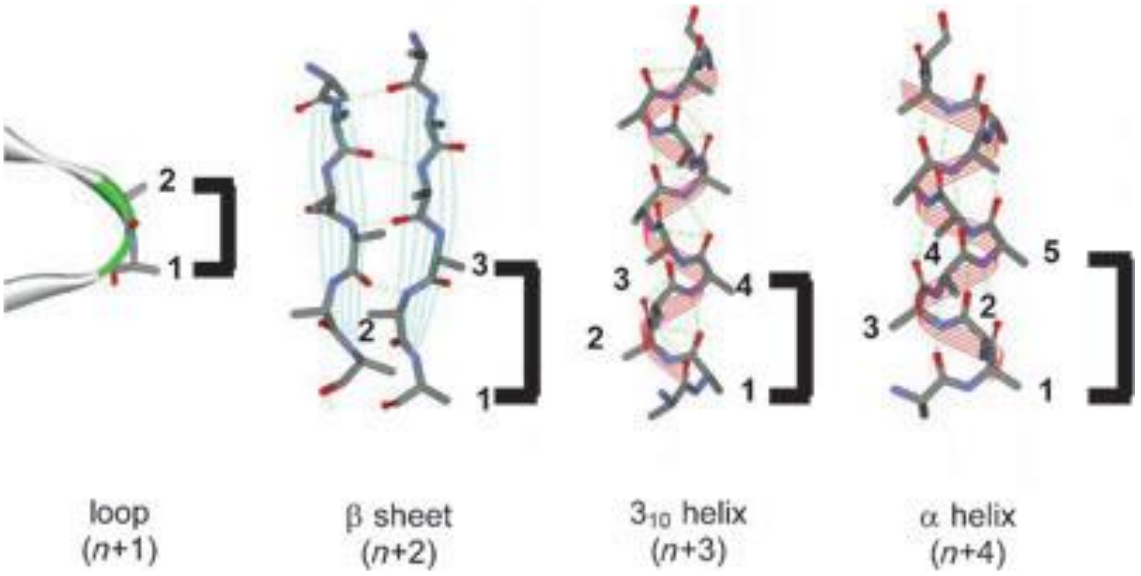


Figure 1.2. Secondary structural guides used by Reetz and co-workers while applying CASTing to create mutant enzyme libraries. (Source: Reetz et al., 2005)

Table 1.1. Degenerate Codons to Introduce Mutations and Their Encoded Amino Acids

Degenerate codon	Corresponding base sequence	Encoded codons	Stop codons	Encoded amino acids	Properties
NNN	(A/T/G/C)(A/T/G/C)(A/T/G/C)	64	TAA, TAG, TGA	All	Fully randomized codon
NNK	(A/T/G/C)(A/T/G/C)(G/T)	32	TAG	All	All 20 amino acids
NNS	(A/T/G/C)(A/T/G/C)(G/C)	32	TAG	All	All 20 amino acids
NDT	(A/T/G/C)(A/T/G)(T)	12	No	Phe, Leu, Ile, Val, Tyr, His, Asn, Asp, Cys, Arg, Ser, Gly	Mixture of polar, nonpoar, positive and negative charged residues (Reetz et al., 2008)
NTN	(A/T/G/C)(T)(A/T/G/C)	16	No	Met, Phe, Leu, Ile, Val	Nonpolar residues
NAN	(A/T/G/C)(A)(A/T/G/C)	16	TAA, TAG	Tyr, His, Gln, Asn, Lys, Asp, Glu	Charged, larger side chains
NCN	(A/T/G/C)(C)(A/T/G/C)	16	No	Ser, Pro, Thr, Ala	Smaller side chains, polar and nonpolar residues
RST	(A/G)(G/C)(T)	4	No	Ala, Gly, Ser, Thr	Small side chains

All directed evolution studies require an evaluation step to identify mutant proteins with desired properties. Assessment methods can be classified into two categories; selection-based methods and screening-based methods. In selection-based approaches, mutant proteins from mutant library are tested for a desired biochemical activity. In *in vivo* selection methods, desired properties may increase viability of host

cell, for instance, synthesis of an essential amino acid, nucleotide or detoxification against environmental toxins[8, 14, 15]. Positive selection methods can be done either on agar plates or liquid media, however negative selection methods require microtiter plates in order to backtrack and re-cultivate cultures with desired properties[16]. Phage display is also used as an *in vitro* selection method in which DNA is fused to phage gene expressing coat proteins. Then fused DNA is combined in to a phagemid. Phagemids are phage derived cloning vectors containing origin of replication, fused with desired display protein. Phagemids usually encode only one type of coat proteins[17, 18]. The phage are then subjected to chromatic separation for desired binders [19]. Screening-based methods are based on the turnover rate of the substrate that is calculated via the applied assay. Assays are usually colorimetric or fluorometric measurements. Cell-free activity assays commonly involve growing colonies individually in 96- or 384- well plates followed by lysis and isolation or enrichment of proteins[20]. These assays are often time consuming and costly, thus size of the mutant library is limited to 10^6 mutants. Larger sizes of mutant libraries ($10^7 – 10^{10}$) can be achieved by using *in vivo* screening-based methodologies that do not require lysis of the cells, such as agar plate colony screening, flow cytometric cell sorting and phage display. Throughput of *in vivo* screening methodologies are generally limited by low efficiency of ligation and transformation[8].

1.2.3. Biocatalysis and Biocatalysts

Biocatalysis is the chemical process through which biological catalysts (biocatalysts) perform reactions between organic components. Biocatalysts have a wide range of application areas, most importantly synthesis of fine chemicals and pharmaceutical industry. Advances in biotechnology led scientists to engineer biocatalysts to perform reactions that are difficult to perform with chemical catalysts. A good biocatalyst should have high activity, selectivity, specificity and efficiency additionally should be environmentally friendly. After discovery of cytochrome P450 oxygenases (P450s), their capability of catalyzing oxidation of hydrocarbons with high efficiency and selectivity has gained attraction in biocatalysis area[21, 22].

1.3. Cytochrome P450 Enzymes (P450s)

P450s are heme *b*-containing mono-oxygenase enzymes and first studies on P450s were started in 1954 by Klingenberg[23]. Heme group in P450s consist of Fe^{3+} ion inside a protoporphyrin IX ring and heme group is held in protein via thiol group of conserved cysteine residue[24, 25]. Ferric ion is coordinated in the center by nitrogen atoms of protoporphyrin ring as shown in Figure 1.3. and have an important function in catalysis. P450 enzymes catalyze oxidation of hydrocarbons with high efficiency and selectivity. P450s are involved in wide range of biochemical reactions and found in all kingdoms of life. Most common reaction of P450s is cleavage of heme-bound molecular oxygen and formation of an oxygenated product as shown in Equation 1[21, 26].

Equation 1. $RH + O_2 + 2e^- + 2H^+ \rightarrow ROH + H_2O$

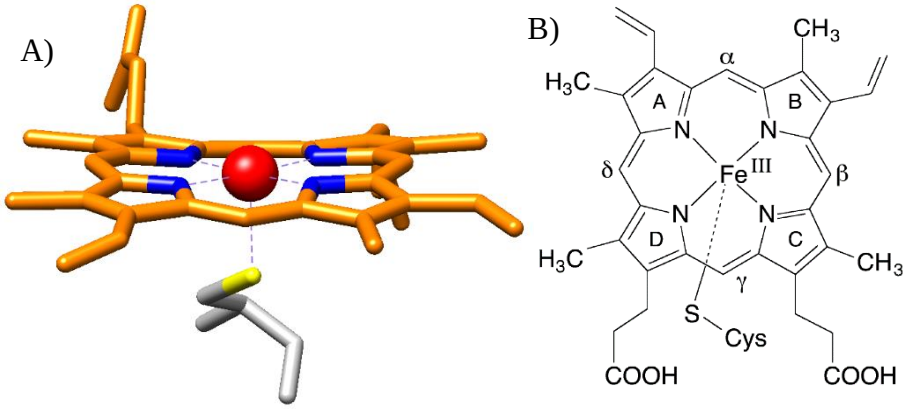


Figure 1.3. A) Illustration of heme group of P450s created in Chimera. Ferric ion (red) is coordinated by four nitrogen atoms (blue) of Protoporphyrin IX ring (orange). Heme group is tethered to protein via thiol group (yellow) of conserved cysteine residue (grey). B) Chemical structure of heme group in P450s (Source: Meunier et al., 2004).

In the equation RH is the substrate and ROH is the oxygenated product. Active site residues carry protons to catalytic center of the P450 in order to cleave molecular oxygen

bond (O–O). Electrons are usually sourced from reduced pyridine nucleotide coenzymes, Nicotinamide Adenine Dinucleotide or Nicotinamide Adenine Dinucleotide Phosphate (NADH or NADPH). In order to transfer electrons from NAD(P)H to the active site, P450s require redox partner proteins[27]. Employment of different cytochrome P450 reductases (CPRs) leads to different P450 systems (Figure 1.4.) with different components. Mitochondrial and most bacterial P450s (class I) are three-component systems, that contains a P450, a ferredoxin and reductase that is NADH-dependent and flavin adenine dinucleotide (FAD) containing. Microsomal P450 (class II) systems have two component systems, a membrane bound P450 and membrane bound NADPH-dependent diflavin reductase (FAD and flavin mononucleotide: FMN). Class III P450s are one component systems that have same cofactors with class II P450s. In contrast to class II, class III P450s are soluble and fused into one polypeptide. In one component enzymes, NADPH-dependent, FMN-containing reductase and ferredoxin are attached to the heme domain of P450s[24, 25, 28].

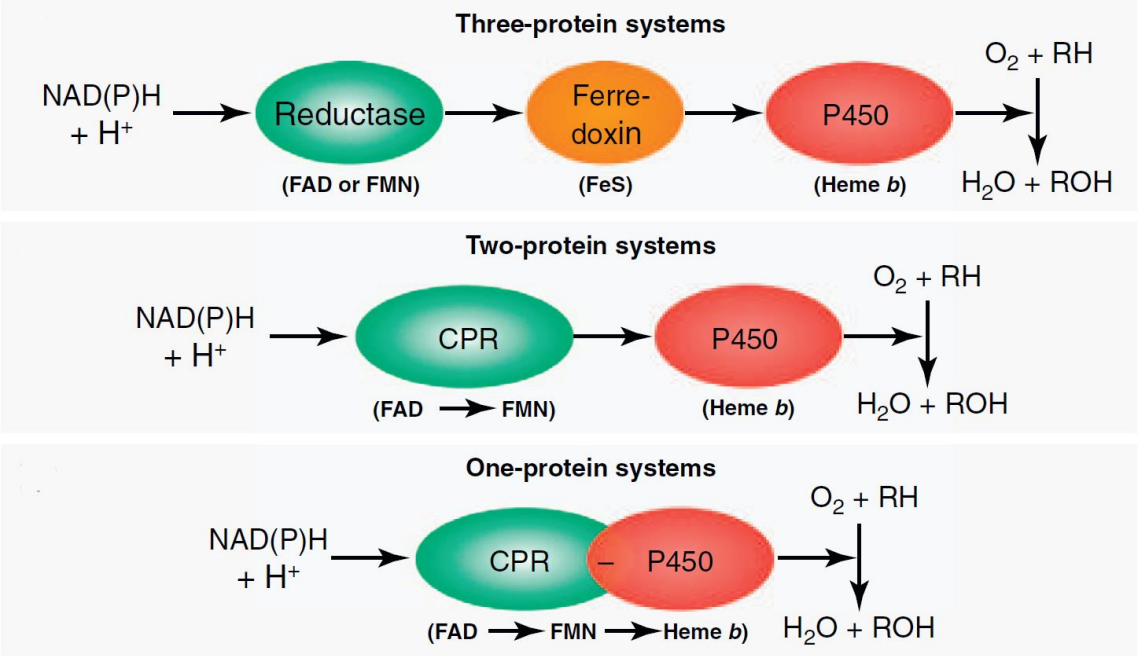


Figure 1.4. General steps involved in reactions catalyzed by P450s. In three protein systems, P450 and reductase can be soluble, or membrane bound. In two-protein systems P450 and CPR are bound to membrane. In one-protein systems, CPR-like reductases are fused to P450s and protein can either be membrane bound or soluble. (Source: Urlacher, V. B., & Girhard, M., 2012)

After substrate (RH) binds to P450s, first of the two electrons obtained from NAD(P)H, reduces the ferric iron ion (Fe^{3+}) of the P450 heme into ferrous form (Fe^{2+}) then ferrous ion can bind dioxygen molecule. After molecular oxygen binds to ferrous ion, second electron transfer occurs and a ferric-peroxy species is produced. Subsequently the ferric-peroxy intermediate is protonated that leads to molecular oxygen cleavage. Cleavage leads to formation of a water molecule and oxyferryl radical cation species that is considered as the main oxidation agent in P450 oxygenation reactions. Oxyferryl species attack to the bound substrate that results into oxygenation of substrate and product formation (ROH). Catalytic cycle of cytochrome P450 is illustrated in Figure 1.5. Additionally, some of the P450 enzymes can catalyze reactions through an alternative pathway called hydrogen peroxide (H_2O_2) shunt. P450 monooxygenases can produce catalytically active oxyferryl species from resting state by using H_2O_2. However, this pathway is relatively inefficient, and H_2O_2 can inactivate the heme-prosthetic group and protein backbone of P450 enzymes[29, 30].

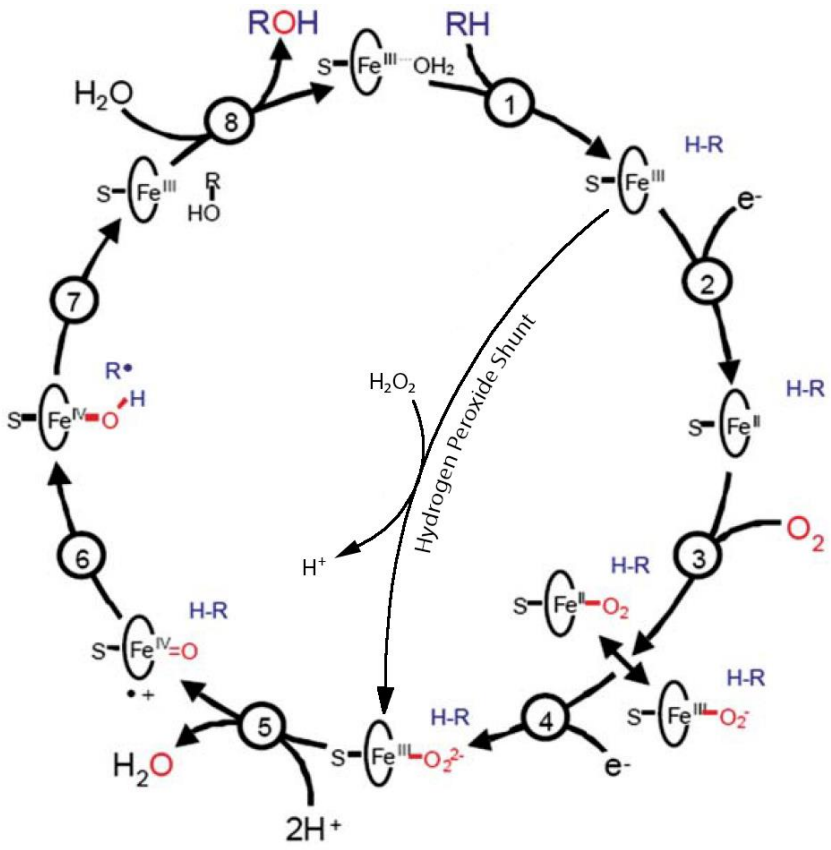


Figure 1.5. Generalized catalytic cycle of cytochrome P450s. (Source: Anderson, J. R., & Chapman, S. K., 2005)

As P450 enzymes are found in variety of organisms, they are involved in many diverse biochemical reactions throughout all kingdoms of life. Examples of basic reactions that involve P450s are C-hydroxylation, dealkylation, deamination, heteroatom oxygenation, heteroatom oxygenation and epoxide formation. In addition to basic reaction P450s also catalyze more complex reactions (Figure 1.7.) such as, chlorine oxygenation, aromatic dehalogenation, ring coupling, ring formation reductive activation, ring contraction, cleavage of amino oxazoles and oxidative aryl migration[31-33]. Moreover, P450s can catalyze oxidation of Amplex Red that is a non-fluorescent reagent. When Amplex Red is oxidized it turns into a red-fluorescent product called resorufin that has excitation maxima at 571 nm and emission maxima at 585 nm (Figure 1.6.)[34-36].

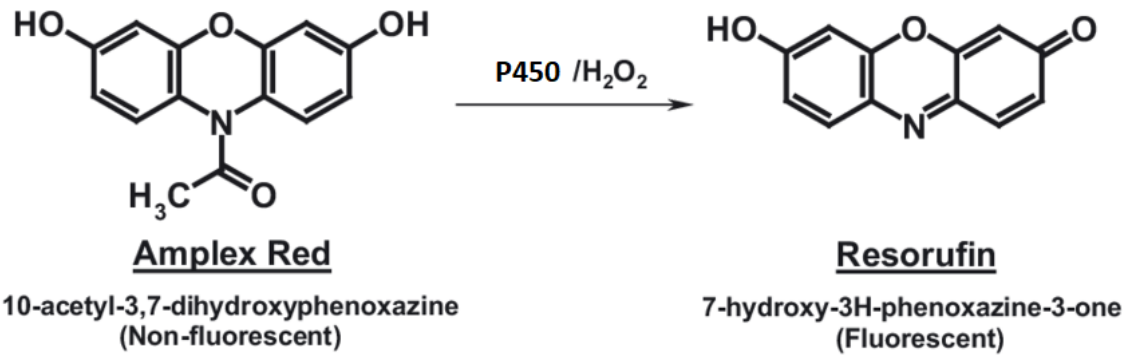


Figure 1.6. Oxidation of Amplex Red into Resorufin catalyzed by P450 by using H_2O_2. (Source: Gorarina & Kulys, 2008)

Addition to diverse reactions of P450 enzymes, they have a wide range of substrate such as, fatty acids, *n*-Alkanes, terpenes, steroids, cholesterol, eicosanoids and vitamins as shown in Figure 1.8.

Having diverse substrate range and involving in catalysis of various reactions gave P450s an important role in many industries such as, pharmaceutical industry (P450s are involved in drug metabolism), physiology (catalyzing synthesis of cholesterol, steroids and fatty acids), agriculture (insecticide detoxification) and biotechnology (protein engineering studies).

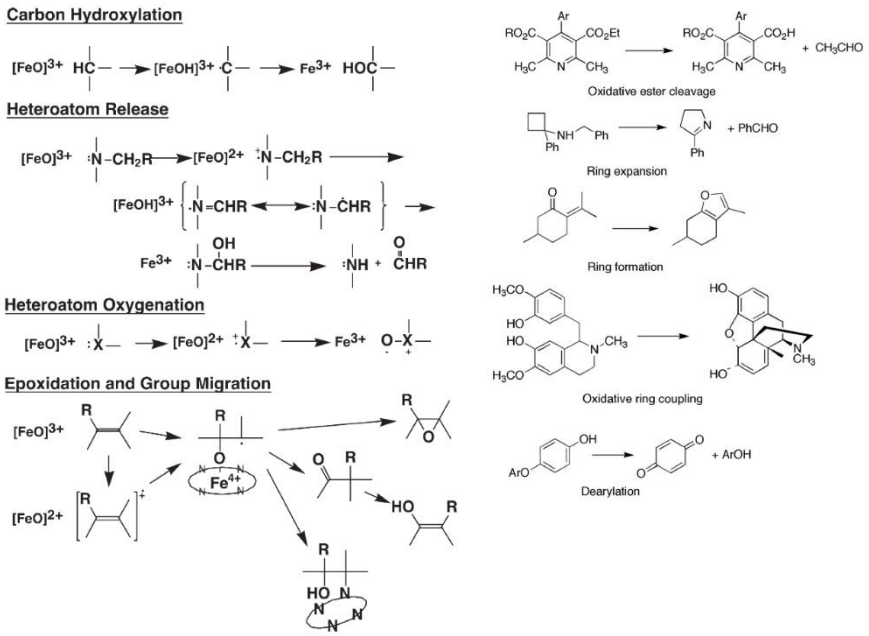


Figure 1.7. Basic (left) and more complex (right) reactions catalyzed by P450s (Source: Isin, E. M., & Guengerich, F. P. 2007).

1.4. Cytochrome P450 119 (CYP119)

CYP119 is a thermophilic enzyme from P450 protein family. At first origin of CYP119 was thought to be *Sulfolobus solfactaricus* by Wright and co-workers in 1996[37]. However, Rabe and co-workers discovered that origin of CYP119 is actually *Sulfolobus acidocaldarius*. Wright and co-workers were misled by contamination of *S. solfactaricus* P1 strain with *S. acidocaldarius*[38]. Among P450s, CYP119 is the first enzyme that was discovered in archaea. *S. acidocaldarius* is a Sulphur-oxidizing organism that grows optimally between 78 and 86 °C, with a range of pH 2-3[39]. Length of CYP119 is 368 residues. Being stable towards high temperature, low pH and having relatively short chain differs CYP119 from other enzymes in P450 family[40]. Crystallization and preliminary X-ray diffraction of CYP119 was first done by Park and co-workers in 2000 , followed by determination of crystal structure of CYP119 in an independent study by Yano and co-workers in 2000[41, 42]. Crystal structure of CYP119 is shown in Figure 1.9. Heme cofactor in CYP119 is tethered to active site of enzyme by thiol group of conserved Cys317 residue. Although natural substrates and CPR of CYP119 are unknown, studies shown that CYP119 can catalyze several reactions such as, styrene epoxidation, lauric

acid hydroxylation, chemical reduction of nitrite, nitric oxide and nitrous oxide, and electrochemical dehalogenation of CCl_4[38, 43].

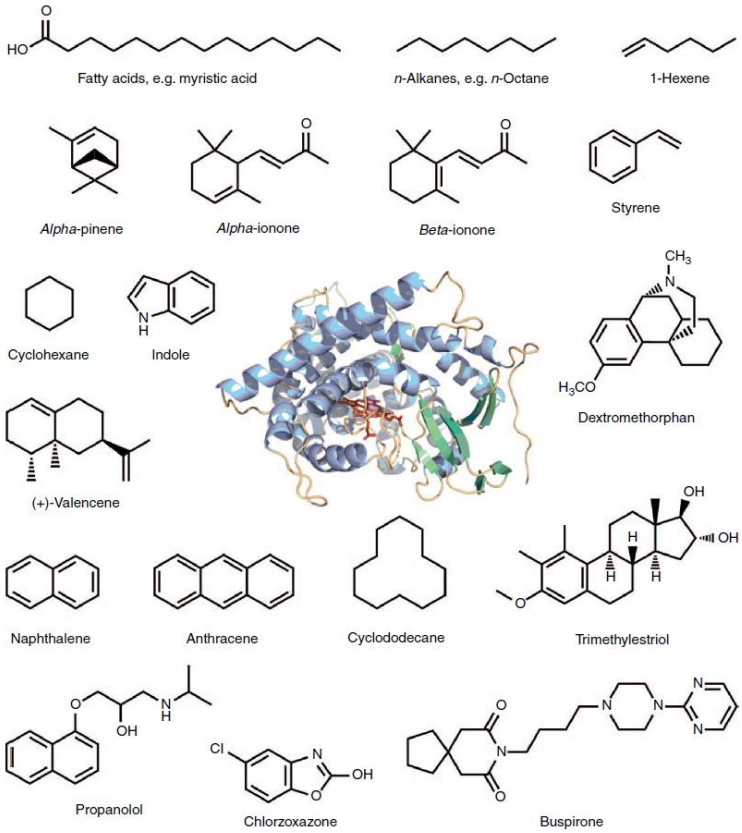


Figure 1.8. Examples for substrates of Cytochrome P450 enzymes.
(Source: Urlacher et al. 2012)

CYP119, a water soluble and thermostable enzyme, exhibiting monooxygenase, oxidoreductase and peroxidase activities has attracted attention and CYP119 was subjected to enzyme engineering studies. Additionally, CYP119 can also utilize hydrogen peroxide shunt pathway. One of the first studies on CYP119 was done by Koo and co-workers, by performing site specific mutagenesis of Thr213 and Thr214 residues which are close to iron atom in heme group as shown in Figure 1.10[45]. Mutagenesis showed that Thr213 residue is catalytically significant and Thr214 residue involves in controlling the spin state of iron in heme.

Effects of substituting Thr213 and Thr214 was shown by using aryldiazene probes. When Thr213 substituted with smaller residues, reaction rate was increased, while larger residues decrease the rate of formation of the aryl-iron intermediate. Substitutions of Thr214 have little effect on the rate of formation, however Thr214 has a significant role in equilibrium of spin state supporting that Thr214 residue affects distal water ligand by polar effects or through hydrogen-bonding network instead of direct hydrogen bonding[44]. In 2002, Koo and co-workers studied on enhancing the hydroxylation of lauric acid by performing site-directed mutagenesis on CYP119. Hydroxylation activity was increased by 15-fold by T214V and D77R mutations. Despite Thr214 being in the active site, Asp77 is located at the binding site for redox partner putidaredoxin. T214V mutation facilitates the binding of substrate and enhances the spin state change thereby increasing the rate of formation. D77R mutation led to improvements in binding of redox partner to CYP119 and increase in the rate of electron transfer from redox partner putidaredoxin to heme group[45]. In recent studies, Hartwig and co-workers replaced the heme group in CYP119 with iridium containing Ir(Me)-mesoporphyrin IX. Variants of CYP119 containing iridium instead of iron had improved rate of catalyzation of carbenes into C–H bonds with up to 98% enantiomeric excess[46, 47]. In 2017, Hartwig and co-workers focused on several hotspots that might affect the activity of CYP119 towards various ethyl 2-diazo-2-(2-methoxyphenyl) acetate derivatives. Starting with a double mutant (stated as CYP119(+); C317G, V254A) and triple mutant (stated as CYP119(-); C317G, L69F, T213V) additional mutations at residues Val254, Ala152, Leu155, Phe310, and Leu318 were investigated (Figure 1.11). As a result, CYP119(+)-L155W variant exhibited 98% enantioselectivity to *cis*-cyclopropane (1S,2R) with 80% yield and CYP119(-)-V254L gave opposite enantiomer (1R,2S) with 98% enantioselectivity and had 74% yield[47, 48]. ,

Addition to the previously studied residues, especially Thr213-and Thr214 residues, when active site residues were examined, Val151-Phe153 residues are close to heme iron and those residues are spatially close to each other as shown in Figure 1.12, allowing CAST method to be applicable. Systematic mutations on Val151-Phe153 residues by applying CAST might result in an improvement in the peroxidation activity of CYP119.

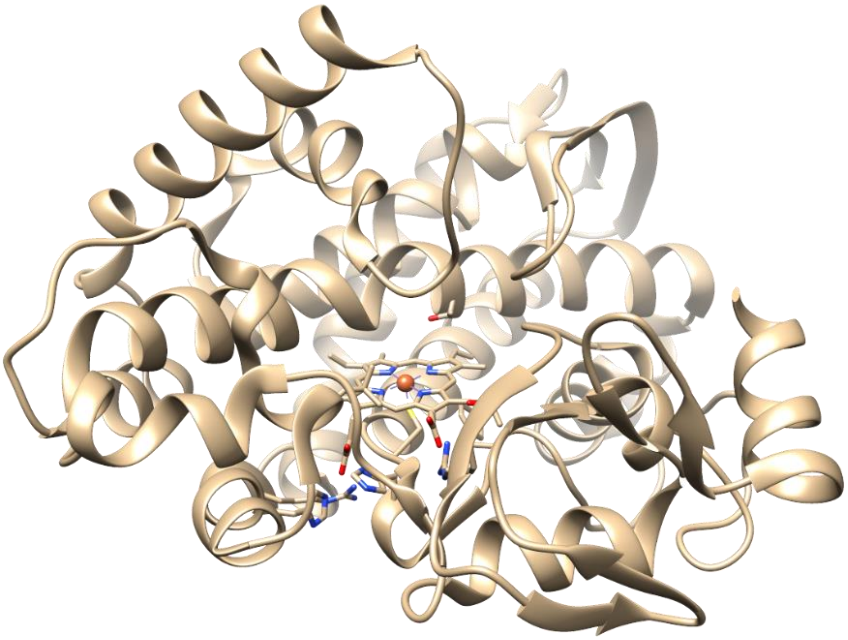


Figure 1.9. Crystal structure of CYP119 created in UCSF Chimera.

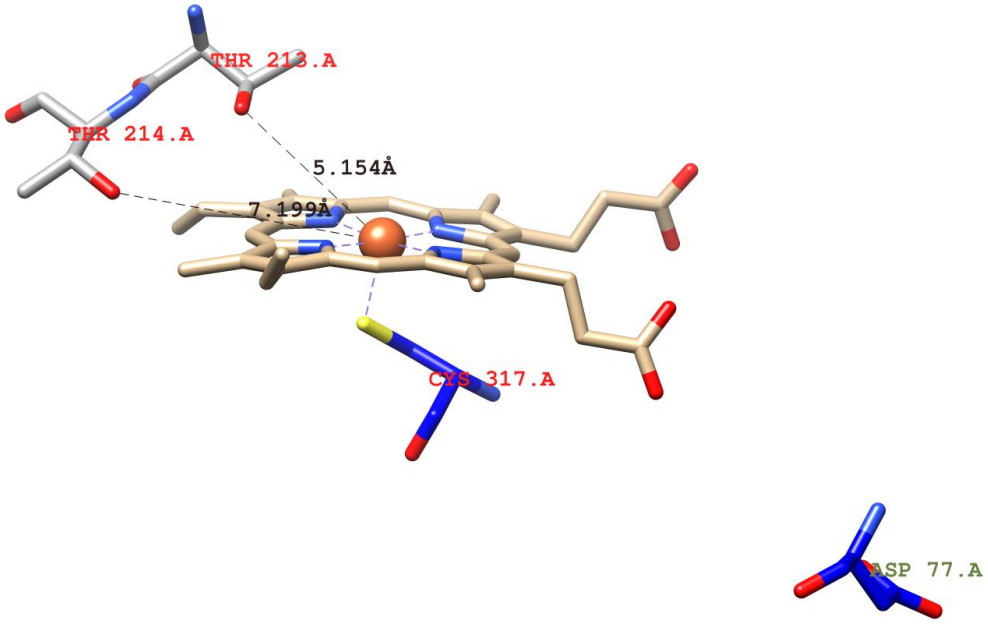


Figure 1.10. Distances of Thr213 Thr214, and Asp77 residues, Koo and co-workers studied on, to heme iron. Illustrated by using UCSF Chimera.

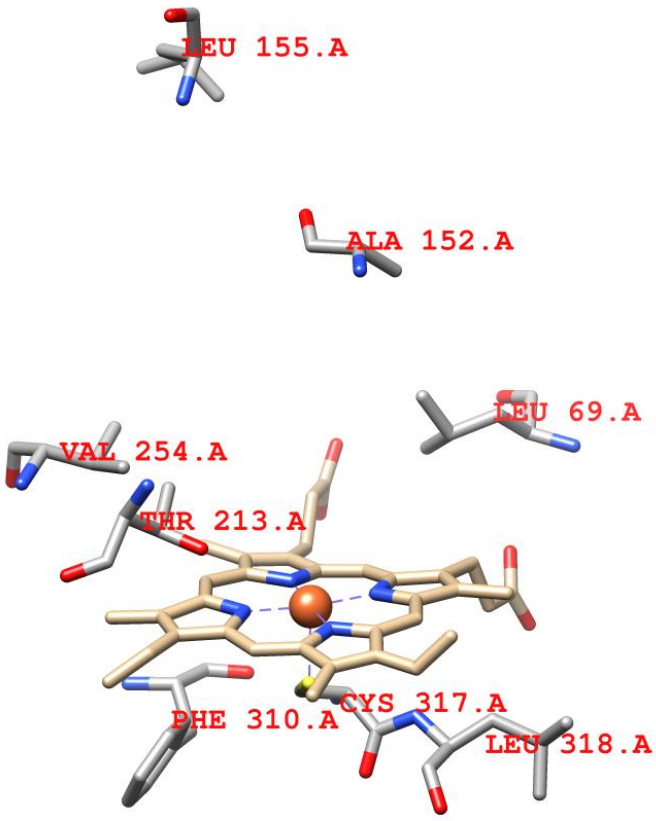


Figure 1.11. Distances of, and Leu69, Ala152, Leu155, Thr213, Val254, Phe310, and Leu318 residues, Hartwig and co-workers studied on, to heme iron. Illustrated by using UCSF Chimera.

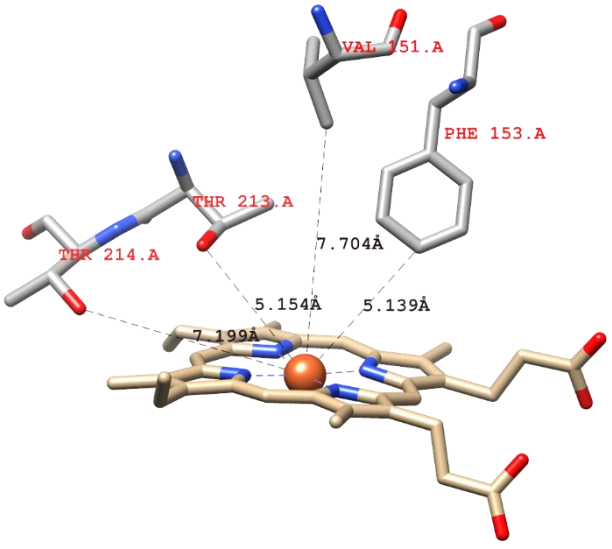


Figure 1.12. Distances of Thr213-Thr214 and Val151-Phe153 residue pairs to heme iron.

1.5. Scope of This Study

In this study, peroxidation activity of CYP119 from *Sulfolobus acidocaldarius* was improved through iterative rounds of directed evolution by applying CAST method. CAST method involves substituting two amino acids that are spatially close to each other to generate synergistic conformational effect on the activity of the enzyme. Substitutions at Thr213 – Thr214 and Val151 – Phe153 residues were introduced by using degenerate primer mixes containing NDT codons. NDT codons contain total of 12 codons that encodes mixture of polar, non-polar, positively and negatively charged amino acid codons which reduces the library size. Created CYP119 mutant library with the mutant genes were screened for improved peroxidation activity toward Amplex Red in the presence of H_2O_2 as an electron donor. Naturally electrons are donated by NAD(P)H which is an expensive reagent for industry. Before screening the mutant CYP119 enzymes, optimizations and tests were done on the assay that used to assess peroxidation activity. After the assay was optimized, screened mutant CYP119 enzymes were compared to WT CYP119 enzyme for improved peroxidation activity. Improved CYP119 mutant enzymes were identified and improved peroxidation activities were further confirmed with larger scale expressions of mutant enzymes and sequence analysis. Effects of the substituted amino acids at the Thr213 – Thr214 and Val151 – Phe153 active site residues on peroxidation activity were analyzed by employing an Amplex Red peroxidation activity assay. With the help of replacing costly NAD(P)H with inexpensive alternative H_2O_2, eliminating the requirement of cytochrome P450 reductase (CPR) and improved peroxidase activity, engineered CYP119 enzymes can be used in large scale applications in pharmaceutical industry. Additionally, since both natural substrate and redox partner of CYP119 is unknown, this study will help enlightenment of CYP119 mechanism.

CHAPTER 2

MATERIALS AND METHODS

2.1. Creation of Mutant Genes by PCR

The plasmid containing Wild Type (WT) CYP119 was gifted by Teruyuki Nagamune (Addgene plasmid #66131)[49] Mutant genes were created by using Q5 Site-Directed Mutagenesis Kit (New England BioLabs Inc., E0554S) and protocol of the kit (E0554) was followed. A total of 25 µL PCR reaction setup is given in Table 2.1. PCR reaction conditions are given in Table 2.2. pET11a containing WT CYP119 gene was used as template DNA. Sequences of, NDT codon containing, degenerate primers that lead substitutions at Thr213-Thr214 and Val151-Phe153 residues are given in Table 2.3. Primers were synthesized by Sentegen Biotech (Ankara, Turkey).

Table 2.1. Reagents used in 25 µL PCR reaction.

Ingredients	**Volume**	**Final Concentration**
Q5 Hot Start High-Fidelity 2X Master Mix	12.5 µL	1X
10 µM Forward Primer	1.25 µL	0.5 µM
10 µM Reverse Primer	1.25 µL	0.5 µM
Template DNA (1-25 ng/µL)	1 µL	1-25 ng
Nuclease-free water	9.0 µL	

The success of PCR run was confirmed by 1% Agarose gel electrophoresis. 1 g of Agarose (Prona Nu Micropor Agarose) was dissolved in 98 mL ddH_2O then 2 mL of 50x TAE buffer was added. TAE buffer contains 2 M Tris base (VWR 201-064-4), 50 mM Ethylenediaminetetraacetic acid (EDTA, Bioshop Canada Inc. EDT001.1), 1 M Acetic Acid (Sigma Aldrich, 27225) dissolved in ddH_2O. After boiling agarose gel mixture, 10

µL of 10,000X SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, S33102) was added to mixture and the mixture was poured onto the plate and comb was placed. While waiting for gel to solidify, DNA samples were prepared. 2 µL of Gel Loading Dye Purple (6X, New England BioLabs, B7024S) was added to 10 µL of DNA sample. For ladder, 1 µL of dye and 4 µL of ddH2O was added to 1 µL of 1kb ladder (New England BioLabs, N3232S). When the gel solidified, tank (Cleaver Scientific Ltd., MSMINIDUO) was filled with 1X TAE buffer. After samples were loaded into wells, agarose gel electrophoresis was run at 110V (Bio-Rad, PowerPac Basic) until dye reaches to end of the gel. Agarose gel was visualized by using Cleaver Safeview-Mini2.

Table 2.2. PCR reaction conditions.

Step		**Temperature (°C)**	**Time (seconds)**
Initial Denaturation		98	45
28 cycles	**Denaturation**	98	10
	Annealing	58	30
	Extension	72	202 (3m 22s)
Final Extension		72	120
Hold		4	∞

Table 2.3. Sequences of Thr213-Thr214 and Val151-Phe153 forward and reverse primers.

Thr213-Thr214	Forward Primer	Reverse Primer
	5’ - GGG TAA TGA GND TND TAC TAA CTT AAT ATC AAA CTC TGT TAT TG - 3’	5’ - GCT ATG AGA AGT AAA ATA ATG TAT C - 3
Val151-Phe153	Forward Primer	Reverse Primer
	5’ - AND TAG GTT GGG TAA GCC TGG A – 3’	5’ - GCA HNT AAG TCT GAC CAC TCT TTG AAC – 3’

Complete removal of template DNA and circularization of newly amplified DNA was achieved by treating PCR product with Kinase-Ligase DpnI (KLD) enzyme mix (New England BioLabs, M05554S) for 30 mins at room temperature (RT). KLD enzyme

mix allows phosphorylation by kinase, intermolecular ligation/circularization of PCR product by ligase and degradation of template DNA by DpnI. KLD treatment reaction mixture are given in Table 2.4. After KLD treatment only mutant genes were left in the PCR tube.

Table 2.4. Reagents used in KLD reaction mixture.

Reagents	Volume	Final Concentration
PCR product	1 µL	
2X KLD Reaction Buffer	5 µL	1X
10X KLD Enzyme Mix	1 µL	1X
Nuclease-free Water	3 µL	

2.2. Preparation of Competent *E. coli* BL21 (DE3) Cells

The Inoue Method[50] was followed to prepare ultra-competent *E. coli* BL21 (DE3) cells. A single *E. coli* BL21 (DE3) colony was picked from a plate that was grown overnight at 37 °C. 25 mL of LB broth (Duchefa Biochemie, LB Broth low salt) was inoculated with the picked colony and incubated for 8h at 37 °C. After incubation, three 1 L flasks containing 250 mL of Super Optimal Broth (SOB) pH 7.0, containing 5g Tryptone (OXOID, LP0042), 1.25 g Yeast Extract (Merck, 1.03753) 125 mg NaCl (Sigma-Aldrich, 31343), 2.5 mM KCl (Sigma-Aldrich, 12636) and 2 mM $MgCl_2$ 8Merck 1.05833) were inoculated with 250 µL, 500 µL and 1000µL of starter culture. Al three flasks were incubated overnight at 18 °C with 220 RPM shaking. OD_{600} values of all three flasks were measured in the following morning and monitored every 45 mins. When OD_{600} value reaches to 0.55, the flask was transferred to the ice water bath for 10 mins. After that 50 mL of cells were harvested by configuration at 3900 RPM (Gyrozen 1580R with GRS-G-750-4 rotor) for 10 minutes at 8°C. the medium was poured off and the tube was dried on paper towels. Cells were resuspended by using 16 mL of ice-cold Inoue Transformation buffer containing 55 mM Manganese (II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$), 15 mM Calcium chloride dihydrate ($CaCl_2 \cdot 2\ H_2O$), 250mM Potassium chloride and 0.5 M pH 6.7 piperazine-1,2-bis(2-ethanesulfonic acid) (PIPES) in dH_2O by

swirling the tube. Cells were harvested by centrifugation by at 3900 RPM at 8°C and medium were poured off. Pellet was resuspended by using 4 mL of Inoue Transformation Buffer and 300 µL of DMSO was added. The tube was incubated on ice for 10 mins. 50 µL aliquots of suspension were transferred into sterile, chilled microcentrifuge tubes and tubes were snap-frozen by using liquid nitrogen. Aliquots were kept in -80°C freezer.

2.3. Construction of Mutant CYP119 Library

Mutant library was constructed by using heat-shock transformation of *E. coli* BL21 (DE3) competent cells with mutant genes, a modified version of Q5 Ste-Directed Mutagenesis Protocol was followed. In order to conduct transformation, 10 mL culture tube was chilled on ice, 50 µL of competent cells were transferred into culture tube. Then 5 µL of KLD mixture was added to competent cells. tube was incubated on ice for 30 mins. After incubation heat shock was applied at 42 °C for 45 secs and cells were incubated on ice for 2 mins. 950 µL of SOC medium (SOB medium with additional 20mM glucose, Sigma Aldrich, 16301) was added to tube. After incubation for 1 hour at 37 °C with 220 RPM shaking, cells were pelleted at 3900 RPM for 10 mins. 800 µL of media was taken and pellet was resuspended with the remaining media. LB agar plate containing 100 µg/mL ampicillin was inoculated with cells and grown overnight at 37 °C, thus mutant library was created.

2.4. Confirmation of Mutant Library

Mutant library was confirmed by sequence analysis of plasmids from three randomly picked colonies. Three randomly picked colonies were grown in 7 mL of LB containing 100 µg/mL ampicillin at 37 °C with 220 RPM shaking for overnight. In the following morning plasmids of the colonies were isolated by using Presto Mini Plasmid Kit (Geneaid, PDH100). Cells were harvested by centrifugation at 3900 RPM for 15 mins. Media was discarded and pellet was resuspended in 200 µL of PD1 buffer containing

RNase and suspension was transferred in a microcentrifuge tube. Cell lysis was done by adding 200 µL of PD2 buffer and inverting the tubes 10 times followed by an incubation for 2 mins at RT. Then 300 µL of neutralization buffer PD3 was added to tubes and mixed by inverting the tubes 10 times. Tubes were centrifuged ay 15,000 x g for 8 mins, then supernatants were transferred into PDH columns inside the 2 mL collection tubes. Columns were washed with 600 µL of wash buffer and centrifugation at 15,000 x g for 3.5 mins. Then columns were transferred into fresh 1.5ml microcentrifuge tubes. Plasmids were eluted by adding 50 µL of Elution buffer to columns and centrifugation at 15,000 x g for 2 mins.

Concentration of isolated plasmids were measured by using Thermo Fisher Scientific Multiskan GO (10680879) and µDrop Plate (Thermo Scientific, N12391). Concentrations of plasmids were adjusted for sequencing requirements and sent to Triogen Biotech for sequencing.

2.5. Optimization of the Amplex Red Peroxidation Activity Assay

Amplex Red peroxidation activity assay was optimized to obtain best possible results. Following tests and modifications were done to assay.

2.5.1. Selection of Blank Used in the Amplex Red Peroxidation Assay

Three different blank samples were tested, each well contains 100 µL of samples, samples were prepared in black 96-well microplates (Thermo Fisher Scientific, 237105). Samples were 100 µL ddH_2O, 50 µL SoluLyse (protein extraction reagent, Genlantis, L100125) mixed with 50 µL pH 7.4 potassium phosphate (KPi) buffer, and 100 µL of pH 7.4 KPi buffer. pH 7.4 KPi buffer was prepared by mixing 80.2 mL of 1 M K_2HPO_4 (Merck, 105101) and 19.8 mL of KH_2PO_4 (Sigma Aldrich, V000225), pH was confirmed by using pH meter (J. P. Selecta, pH-2005)

Amplex Red mixture used in the Amplex Red assay consists of 20 µM Amplex Red (Thermo Fisher Scientific, A12222), 2 mM H_2O_2 in 50 mM pH 7.4 KPi buffer. A total of 100 µL of reaction volume contains 50 µL of WT CYP119 SoluLyse extracted enzyme was mixed with 50 µL of Amplex Red mixture. After ten minutes of incubation at RT, raw values of samples were compared to fluorescence emission created by resorufin which is a product of Amplex Red in the presence of WT CYP119. Measurements were taken by using FLUOstar Omega (BMG LABTECH) equipped with fluorescence filters. Excitation was done by using 545 nm wavelength and emission was measured at 595 nm wavelength.

2.5.2. Addition of EDTA to the Amplex Red Mixture

Negative controls used in Amplex Red assay were exhibiting high values when compared to activity of WT CYP119 in the activity assay. This might be resulting from abundant Fe^{2+} ions found in the ultra-pure water. Fe^{2+} ions can oxidize non-fluorescent Amplex Red to fluorescent resorufin. In order to chelate metal ions in ddH_2O a final concentration of 1 mM chelating agent Ethylenediaminetetraacetic acid (EDTA) was added to Amplex Red mixture. Values of negative control samples with EDTA and without EDTA was compared to fluorescent emission value from resorufin created by WT CYP119.

2.5.3. Testing the Sensitivity of the Amplex Red Assay

In order to determine sensitivity of fluorescent detecting FLUOstar Omega microplate reader, an experimental setup as shown in Table 2.5. was used. Each well contains a total of 100 µL reaction volume that contains varying concentrations from 0 µLM to 10 µM of Amplex Red, 1 mM H_2O_2 in KPi buffer and 1 µUnit of Horseradish Peroxidase (HRP). Conversion of Amplex Red to resorufin was obtained by addition of 1 µUnit of HRP to each sample. EDTA containing samples contain a final concentration

of 0.5 mM EDTA. Fluorescent emission values of samples were measured with FLUOstar Omega at 595 nm, each sample was excited at 545 nm.

Table 2.5. Experimental setup for sensitivity test

Samples	Amplex Red concentration (µM)	EDTA
1	0	-
2	0.5	-
3	1	-
4	2	-
5	5	-
6	10	-
7	0	+
8	0.5	+
9	1	+
10	2	+
11	5	+
12	10	+

2.5.4. Effect of Incubation Time on the Amplex Red Assay

In order to determine effect of incubation time on Amplex Red assay two WT CYP119, two pET20b and eight CYP119 mutants were grown in 5 mL LB containing 100 µg/mL ampicillin overnight at 37 °C w 220 RPM shaking. In the following day 30 µL from each starter culture were transferred to 8 mL 2xYT media (for 1L, 16 g tryptone, 10 g yeast extract and 5 g sodium chloride (NaCl) are dissolved in dH_2O) containing 100 µg/mL ampicillin and incubated at 37 °C with 220 RPM shaking. When OD_{600} reached to ~ 0.8, Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to each tube with a final concentration of 1mM to induce protein expression and cultures were continued to be incubated for 30 hours at 30 °C with 220 RPM shaking. After 30 h incubation, 3 mL of each cell culture was harvested and kept in -80°C. Pellets were resuspended in 300 µL

of SoluLyse protein extraction reagent and incubated for 10 mins at room temperature (RT) with 220 RPM shaking. Samples were centrifuged at 15,000 RPM for 10 mins to precipitate cell debris and enrich proteins in the supernatant. After centrifugation supernatants were transferred to a clean tube.

Amplex Red assay was performed with 50 µL of each SoluLyse extracted protein. In the original assay incubation time was 10 mins. In order to investigate the effect of incubation time on the Amplex Red assay, fluorescent emission values of each sample were measured with 2 minutes intervals for 20 minutes, after 10 mins of incubation, Fluorescence emission values of samples through 30 minutes time were analyzed.

2.5.5. Effect of Freezing Extracted Proteins Before Amplex Red Assay

In order to determine effect of freezing proteins before Amplex Red assay, two identical experimental sets were setup. Each set contains 2 WT CYP119, 2 pET20b and 9 CYP119 mutants, colonies were grown in 5 mL LB containing 100 µg/mL ampicillin overnight at 37 °C with 220 RPM shaking. In the following day 30 µL from each starter culture were transferred to 8 mL 2xYT media containing 100 µg/mL ampicillin and incubated at 37 °C with 220 RPM shaking. When OD_{600} reached to ~ 0.8, IPTG was added to each tube with a final concentration of 1mM to induce protein expression and incubated for 25 hours at 30 °C with 220 RPM shaking. After incubation, 3 mL of each cell culture was harvested and kept in -80°C overnight. Pellets were resuspended in 300 µL of SoluLyse lysis buffer and incubated for 10 mins at RT with 220 RPM shaking. Samples were centrifuged at 15,000 RPM for 10 mins to precipitate cell debris and enrich proteins in the supernatant. After centrifugation supernatants were transferred to a clean tube. After extraction extracted proteins were kept at -20 °C. Nonfrozen proteins were obtained with the same procedure, only difference is extracted proteins were used after SoluLyse extraction.

Amplex Red assay was done with both experimental setups and relative fluorescence activity values were compared between frozen enzymes and freshly extracted enzymes.

2.6. High-Throughput Screening of CYP119 Mutant Library

Screening of mutant CYP119 library was done by inoculating 96-well cell culture plate (VWR, 734-2781). Each well contains 140 µL LB, containing 100 µg/mL ampicillin ampicillin. LB media were inoculated with *E. coli* BL21 (DE3) cells that carry WT CYP119, pET20b and CYP119 mutant plasmids, by picking colonies from LB agar plates with 10 µL pipette tips and leaving the tips dipped into media for 30 secs. 96-well plate was incubated for overnight at 37 °C with 220 RPM shaking. After incubation 3 µL from each culture were transferred into 96 deep well plates containing 500 µL 2xYT media with 100 µg/mL ampicillin, this step is done as triplicate copy. Plates were incubated at 37 °C with 220 RPM shaking until reaching OD_{600} ~0.5 value, protein expression was induced by adding a final concentration of 1 mM IPTG to cells in the two of the copy plates and incubated for 20 h at 30 °C with 220 RPM shaking. Cells in the third copy plate was mixed with 50% glycerol 1/1 volume ratio to create glycerol cell stock and kept in -80 °C for further use of cells. After 20 h incubation, cells were harvested by centrifugation at 15,000 RPM for 5 mins. Media was poured off and plates were dried on towel paper to remove remaining media in the tube. Pellets were stored at -80 °C overnight. Following day 50 µL of SoluLyse was added to each sample and pellets were suspended. Samples in 2 copy plates were combined in a microcentrifuge tube and incubated for 10 mins at RT with 220 RPM shaking. After cells were disrupted, soluble proteins were enriched in the supernatant by centrifuging tubes 15,000 RPM for 10 mins. Supernatants were transferred into clean tubes.

Amplex Red peroxidation activity assay was conducted with the SoluLyse extracted proteins. In Amplex Red assay, each well has a total of 100 µL reaction volume. Blank used in assay was 1:1 v/v SoluLyse:KPi buffer, negative controls were 50 mM pH 7.4 KPi buffer, containing 1 mM H_2O_2, 10 µM Amplex Red, 0.5 mM EDTA and extracted proteins from pET20b plasmid containing *E. coli* BL21 (DE3). Positive control was 1µUnit HRP in Amplex Red mixture. Peroxidase activity of CYP119 mutants were measured by mixing 50 µL of extracted CYP119 enzyme with Amplex Red mixture. After 10 mins of incubation fluorescence emitted by resorufin was measured a 595 nm by using FLUOstar Omega (BMG LABTECH).

FLUOstar Omega is equipped with combination optics. Top optic heads are fluorescent intensity (FI), absorbance (ABS) and time-resolved fluorescent (TRF), and bottom optic head is FI. Equipped fluorescent optical filters are 545-10 filter for excitation at 545 nm wavelength and A595 filter for emission at 595 nm wavelength. In setup, FI method with endpoint mode was used. In optic options top optics were selected, number of multichromatics was selected as 1, excitation filter was 545-10 and emission filter was A595. Settling time was selected as 0.5 s, measurement start time was 0.0 s, and number of flashes per well was adjusted to 20. Gain adjustment was done with automatic gain adjustment before the measurement by selecting positive control.

After measurements were completed, raw values were normalized to fluorescent emission created by WT CYP119, by dividing every raw value to value of WT CYP119. From the relative fluorescent activity data CYP119 mutants that show increased activity was selected.

2.6.1. Confirmation of Improved Activity Mutants

In order to confirm improved activity of the mutant CYP119 enzymes, *E. coli* BL21 (DE3) cells that contain selected improved mutant plasmids were picked from third replicate plate, inoculated into 5 mL LB media containing 100 µg/mL ampicillin and grown overnight at 37 °C with 220 RPM shaking. Then 30 µL of each starter culture was transferred into 8 mL 2xYT media containing 100 µg/mL ampicillin. When cultures reached to OD_{600} ~0.7 IPTG with a final concentration of 1 mM was added to each tube and incubated for 25 h at 30 °C with 220 RPM shaking. After 25 h of incubation 3 mL of cells were harvested by centrifugation at 15,000 RPM for 10 mins. 300 µL of SoluLyse was added to each pellet and cells were resuspended by gently pipetting. Samples were incubated at RT for 10 mins with 220 RPM shaking. Cell debris and insoluble proteins were precipitated by centrifugation at 15,000 RPM for 5 mins. CYP119 enzymes were enriched in the supernatant. Amplex Red assay was done with 50 µL of each extracted protein.

CYP119 mutants that has confirmed improved activity, were selected and inoculated into 7 mL LB media containing 100 µg/mL ampicillin and grown overnight at

37 °C with 220 RPM shaking. Following morning plasmids were isolated with by using Presto Mini Plasmid Kit. Isolated plasmids were sent to Triogen Biotechnology (Istanbul) for sequencing.

2.6.2. Spectroscopic Analysis of Improved CYP119 Mutants

Optical absorption spectra of WT CYP119 and improved CYP119 mutants were measured between 350 nm and 700 nm wavelength. SoluLyse extracted enzymes were diluted with 1/100 ratio with pH 7.4 50 mM KPi buffer in 0.70 mL quartz cuvettes (TQP, TQP-QS-442) and spectra was measured by using spectrophotometer (VWR, UV-1600 PC). UV Absorption spectra of enzymes was examined for Soret peak observations.

2.6.3. Determination of Extracted Protein Concentrations

Concentrations of SoluLyse extracted proteins from bacteria cultures were measured by using Pierce™ Coomassie Plus (Bradford) Assay Kit (Thermo Fisher Scientific, 23236). Standard curve was plotted by using varying concentrations (25 µg/mL, 20 µg/mL, 15 µg/mL, 10 µg/mL, 5 µg/mL, 2.5 µg/mL, 0 µg/mL) of bovine serum albumin (BSA) and using absorbance values at 595 nm wavelength. In assay a total volume of 300 µL contains 150 µL Coomassie reagent and 150 µL sample and mixture was incubated for 10 mins at RT. Protein extracts were diluted 200 times to match the standards. The formula derived from the standard curve was used to calculate the concentrations of extracted proteins from colonies.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Optimization of the Amplex Red Peroxidation Activity Assay

A directed evolution study requires a screening method, in this study peroxidation of Amplex Red was employed.

Amplex Red is a non-fluorescent compound, oxidation of Amplex Red by peroxidase results in formation of the fluorescent compound resorufin. Resorufin emits fluorescence at 585 nm when excited at 571 nm. CYP119 mutant library was screened for improved peroxidation activity of Amplex Red. Developed Amplex Red peroxidation assay involves proteins extracted from *E. coli* BL21 (DE3) bacteria containing mutant CYP119 genes and Amplex Red mixture (1 mM H_2O_2, 10 µM Amplex Red and 1 mM EDTA in 50 mM pH 7.4 KPi buffer). After 10 minutes of incubation in the dark at room temperature, fluorescent emissions of samples were measured with 595 nm fluorescent filter, excitation was done at 545 nm. Mutant enzymes with improved activities were selected.

In order to develop and conduct an effective assay, there are several fundamental properties to take into consideration such as, specificity, sensitivity, dynamic range, interference, robustness, reproducibility, and accuracy. Developed assay should only detect the desired molecule, in order to assure this negative and positive controls should be included to assay. Additionally, the molecule should be detected in different concentrations and concentrations should fall within the range of assay. Components used in the assay should not interfere with assay such as an assayed sample should not quench fluorescent data. In order to collect reliable and usable data, assay should be robust and reproducible. Differences in preparation, handling or individuals operating the assay should not have any effect on the outcome of the assay[51].

In order to effectively screen the created mutant library, Amplex Red assay should be optimized. In the original assay ultra-pure water was being used as a blank however reaction was performed in 50 mM pH 7.4 KPi buffer with SoluLyse extracted proteins. Another issue arose was the high fluorescent emission values at 595 nm of extracted proteins from pET20b containing *E. coli* BL21 (DE3) cells. Furthermore, additional tests regarding incubation time of enzymes with Amplex Red mixture, sensitivity of the measurement device and effect of freeze/thaw cycles on enzymes activity were done. Adjustments and modification done on the assay are discussed below.

3.1.1. Selection of Blank Used in the Amplex Red Peroxidation Assay

Blank solutions are significant for assays to calibrate the instrument and eliminate the signals created by reagents used in preparation of the assay. A blank solution should contain all components of the sample solution except analyte material.

In order to determine the most suitable blank solution for Amplex Red peroxidase activity test, fluorescent emission measurements (excitation at 545 nm, emission at 595 nm) were taken from different blank candidates: ultra-pure H_2O (UPW), KPi buffer and 50 µL SoluLyse + 50 µL KPi solution. As shown in Figure 3.1., 50 µL SoluLyse + 50 µL KPi solution containing every reagent except analytes gave the lowest value.

Amplex Red assay is a fluorometric assay and fluorescent emission values of samples at 595 nm are being compared, consequently blank used in assay should have low emission value due to not containing any fluorescence material. Measurement was done seven times on three identical copies of each sample. Averaged fluorescent emission values, at 595 nm, of samples were, UPW 60.14±19.87; KPi 153.90±22.34; 50/50 SoluLyse/KPi 2±1.48; WT CYP119 153.67±26.58. Thus, 50/50 SoluLyse/KPi selected as blank for further measurements of Amplex Red peroxidase activity assay.

3.1.2. Addition of EDTA to the Amplex Red Mixture

Negative controls were used in the Amplex Red assay to demonstrate what is the outcome if there is no peroxidase activity in reaction media. Thus, proteins extracted from *E. coli* BL21 (DE3) bacteria containing pET20b plasmid was used as a negative control, because transformed pET20b plasmid only contains ampicillin resistance gene and does not exhibit any peroxidation activity. Additionally, 50/50 Amplex Red mixture/KPi mixture were used as another negative control to observe what was the outcome if there was no extracted protein in the reaction media. Even though there were no peroxidation activity observed in the reaction wells, negative controls were showing high fluorescent emission values at 595 nm. Abundant Fe^{2+} ions in solution can also lead to oxidation of the substrate Amplex Red[52, 53]. To lower the background oxidation of substrate the chelating agent EDTA with a final concentration of 1 mM was added to the reaction mixture (10 µM Amplex Red and 1 mM H_2O_2 in 50 µM pH 7.4 KPi buffer) in order to chelate Fe^{2+} ions in the ddH_2O. Thus, the values of negative controls were decreased as shown in Figure 3.2. EDTA was added to the Amplex Red mixture in order to prevent oxidation of Amplex Red by Fe^{2+} ions in ddH_2O_2.

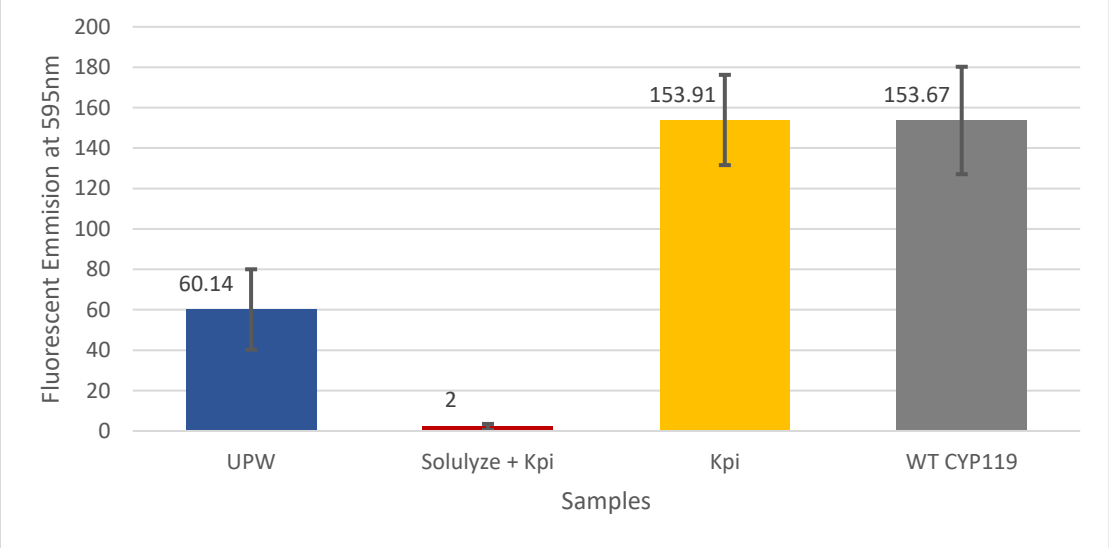


Figure 3.1. Raw data of fluorescence emitted by different blank samples. All wells have a total volume of 100µL, 50 µL SoluLyse + 50 µL KPi solution. WT CYP119 well has 50µL of SoluLyse extracted protein and 1 mM H_2O_2, 10 µM Amplex Red, in 50 µL of KPi buffer (pH 7.4)

3.1.3. Testing the Sensitivity of the Amplex Red Assay

In order to determine the fluorescence sensitivity of the assay, an experiment was setup with varying Amplex Red concentration. Amplex Red was turned into Resorufin by horseradish peroxidase, fluorescence emitted from Resorufin were measured. Emission values are shown in Figure 3.3. and Figure 3.4.

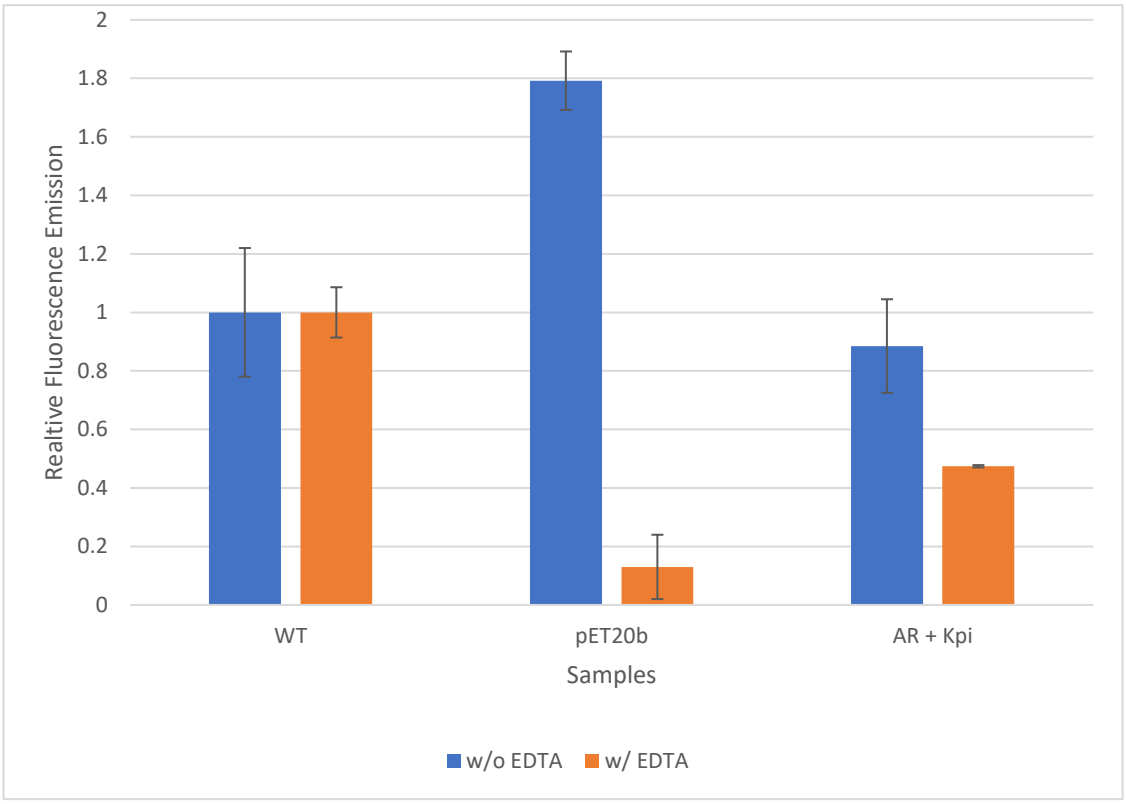


Figure 3.2. Effect of EDTA addition to Amplex Red mixture. A total of 100 µL reaction volume contains 50 µL of SoluLyse extracted enzyme, 2 mM H_2O_2, 10 µM Amplex Red. EDTA included reactions contains EDTA with a final concentration of 1 mM.

As shown in Figure 3.3. and Figure 3.4. FLUOstar by BMG LABTECH can detect fluorescence difference between resorufin created by HRP even at low concentrations such as 0.5 µM and 1 µM Amplex Red. In Amplex Red peroxidation activity test a final concentration of 10µM Amplex Red is used for best possible results.

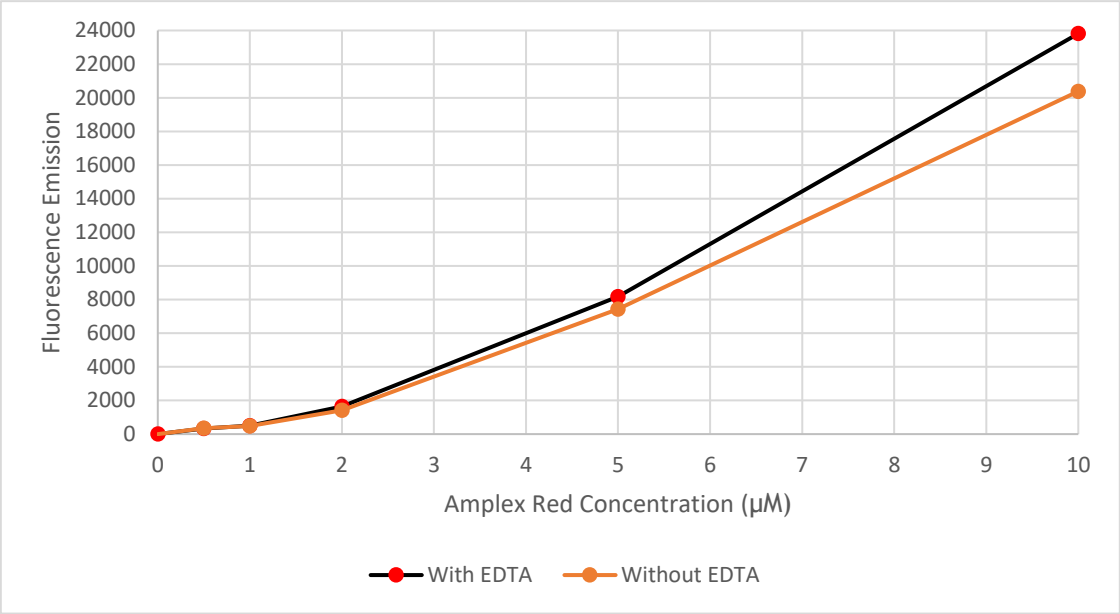


Figure 3.3. Fluorescence emitted by Resorufin created by Horseradish Peroxidase in different Amplex Red concentrations, 0.5 µM - 10 µM. Reaction volume of 100 µL contains 1 µUnit/ml Horseradish peroxidase, 50 µL of SoluLyse and 50 µL of Amplex Red + H_2O_2 in 50 mM pH 7.4 KPi buffer.

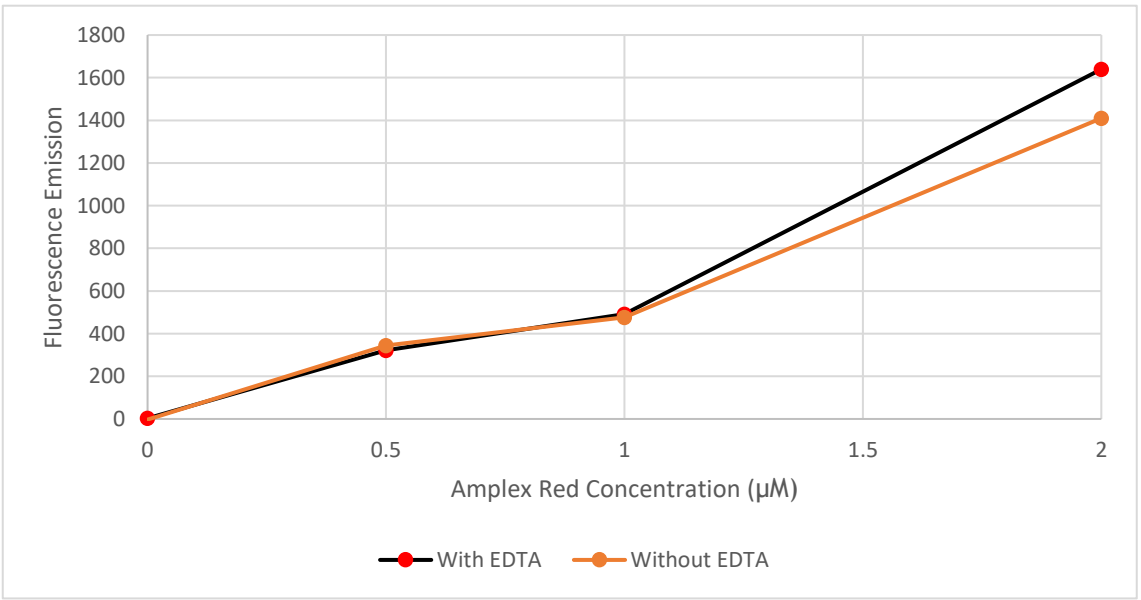


Figure 3.4. Subplot of Figure 3.3. This figure is concentrated on the concentration of Amplex Red between 0 µM and 2 µM.

3.1.4. Effect of Incubation Time on the Amplex Red Assay

Fluorescence emission of an experiment set including WT CYP119, negative controls and eight mutant CYP119 were measured for 30 minutes with two minutes of intervals. Activity assay results were shown in Figure 3.5.

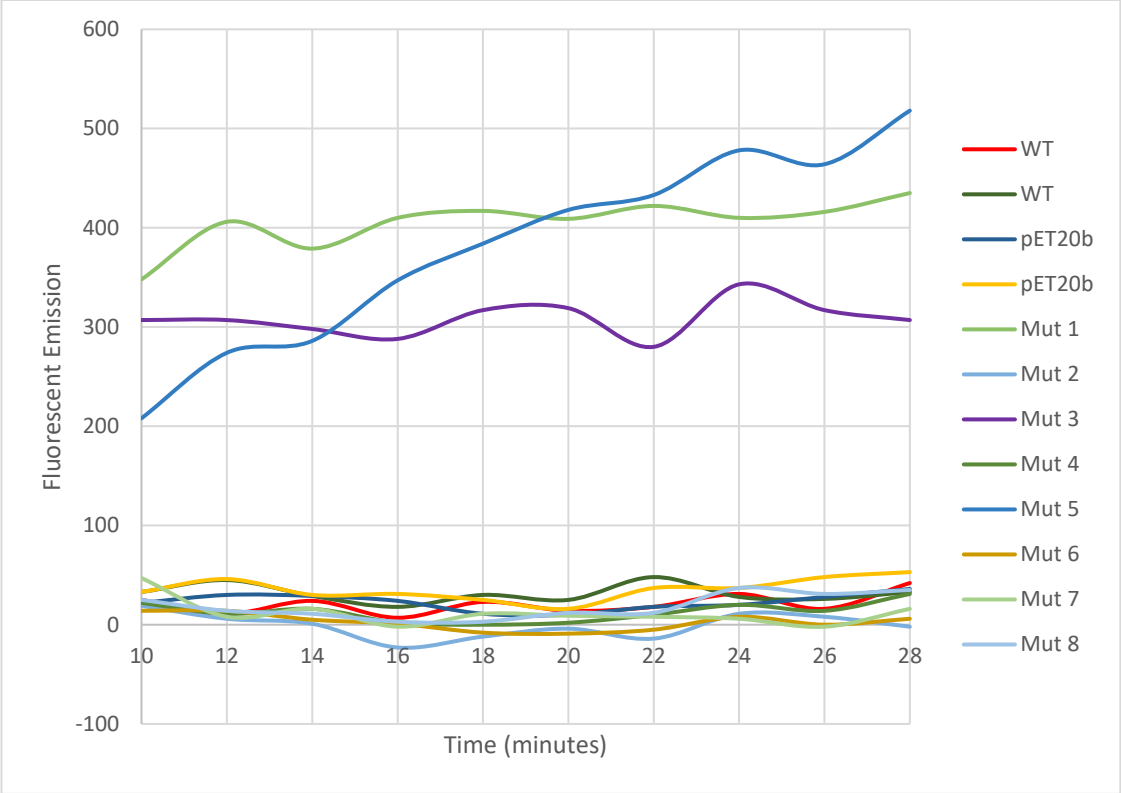


Figure 3.5. Fluorescent activity through time. Measurements were taken after 10 minutes of initial incubation at room temperature (RT), with 2 minutes of intervals for 20 minutes. A total of 100 µL reaction volume contains 2 mM H_2O_2, 10 µM Amplex Red in 50 µL of KPi buffer (pH 7.4) and 50 µL of the extracted enzyme.

Figure 3.5. indicates that incubation time has no significant effect on samples after 10 minutes. Only Mut 5 showed an increase through time and reason for the increase through time is unknown. Incubation time was selected as ten minutes after mixing the Amplex Red mixture with extracted proteins by SoluLyse treatment.

3.1.5. Effect of Freezing Extracted Proteins Before Amplex Red Assay

Previous studies have shown that freezing and thawing cycles can decrease protein activity[54, 55]. Growth of bacteria that contain mutant genes, protein expression in these bacteria and extraction of expressed proteins were completed in identical conditions. The peroxidase activity of freshly extracted and frozen proteins was measured, and their values were compared in Figure 3.6. Measurement was done once on three copies of each sample. As seen in Figure 3.6. that there is no significant difference between freshly extracted and frozen protein activities. There are two mutants M4/24 and B2/15 that exhibit higher activity when frozen proteins compared to freshly extracted ones. The reason for increase in activity of these samples is currently unclear.

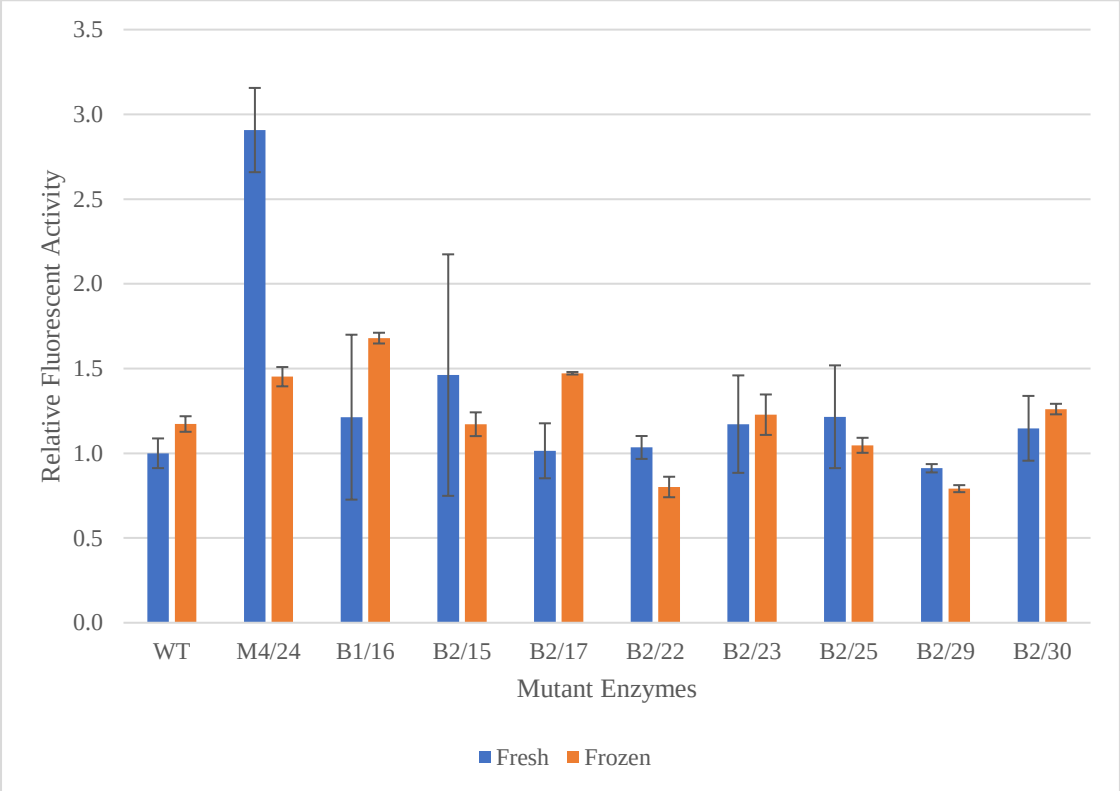


Figure 3.6. Relative fluorescence values of freshly SoluLyse extracted proteins compared with frozen proteins. A total of 100µL reaction volume contains 50 µL of SoluLyse extracted enzyme, 2 mM H_2O_2, 10 µM Amplex Red. EDTA included reactions contains EDTA with a final concentration of 1mM.

3.2. Mutation of CYP 119 at Thr213 and Thr214

Thr213 and Thr214 residues are positioned close to the heme group in the active site of CYP119 enzyme (Figure 3.7.). Previous studies have shown that mutations at these sites lead to changes in enzyme activity[44-47].

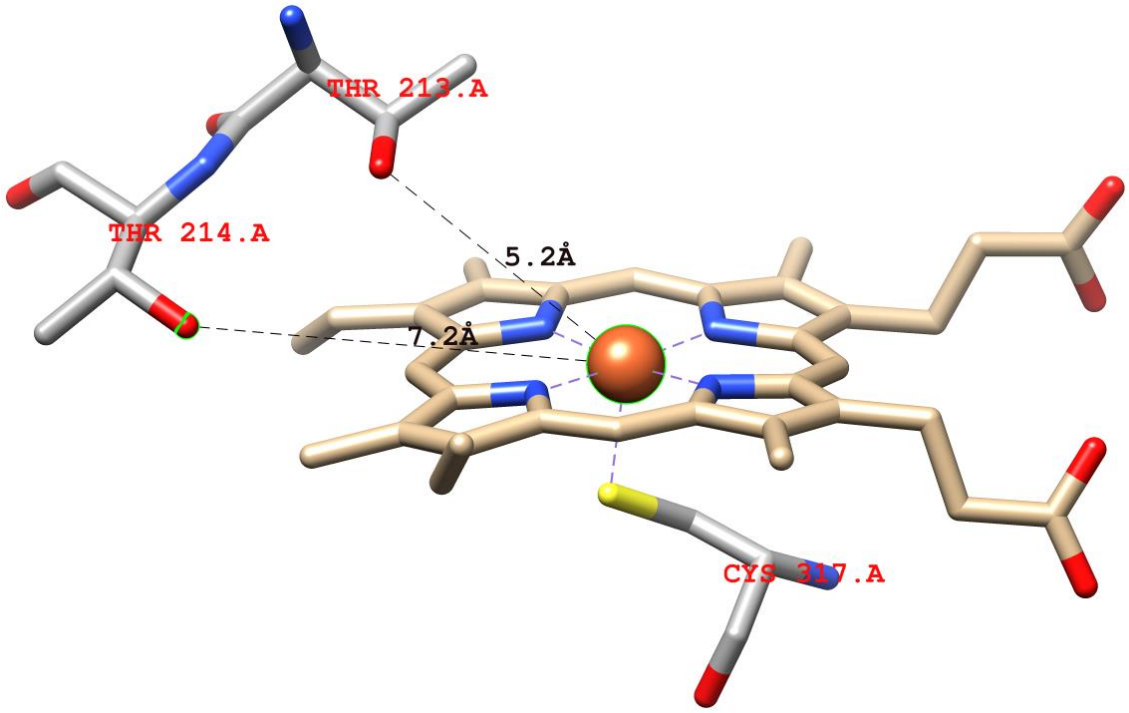


Figure 3.7. Distances of Thr213 and Thr214 residues to heme iron.

3.2.1. Creation of Mutant Genes by Polymerase Chain Reaction

Creation of mutant genes was done by using the CAST method. Polymerase Chain Reaction (PCR) that uses designed degenerate primer mix containing NDT codons create mutations at Thr213 and Thr214 residues in WT CYP119 + pET11a plasmid. PCR products were treated with Kinase-Ligase-DpnI (KLD). After incubation PCR product

with KLD enzyme mix, only mutant genes were left. Confirmation of PCR run was done by running PCR product on %1 agarose gel. Length of CYP119 in pET11a plasmid is 6748 bp. Agarose gel image was shown in Figure 3.8. In the agarose gel image, the band confirms that PCR was done successfully. Ladder used in the gel was mistakenly put 100 bp DNA ladder instead of 1 kb DNA ladder.

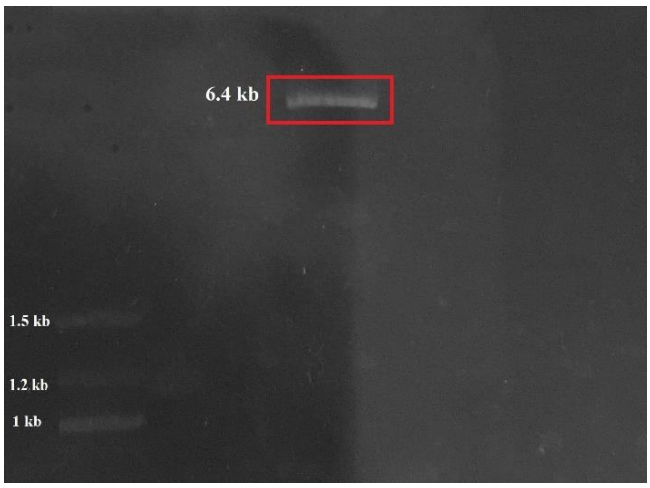


Figure 3.8. Agarose gel image of KLD treated PCR products, length of PCR product (CYP119 in pET11a) is 6.7 kb.

3.2.2. Construction of the CYP119 Thr213 and Thr214 Mutant Library

Mutant library was constructed by transforming mutant genes created in 3.2.1 obtained to high-efficiency competent *E. coli* BL21 (DE3) cells. After growing cells on LB agar plates containing ampicillin overnight at 37 °C, three random colonies were picked from plates for confirmation of mutations. Plasmids sequences of three random colonies were analyzed. Sequence analysis confirmed that plasmids from three random colonies contain mutations at desired sites as shown in Table 3.1. A similar library was constructed by Emre Haklı and frequencies of substituted amino acids and mutational biases were previously determined his work "Generation of Mutant Libraries for Directed Evolution of a Thermophilic P450 Enzyme". 15 colonies were analyzed and there was no

mutational bias observed among them[56]. At Thr213 amino acid position, there were ten possible amino acids out of twelve amino acids and at Thr214 amino acid position, seven different amino acid out of twelve possible substitutions was observed.

Table 3.1. Sequence analysis of plasmids from randomly selected three colonies.

Nucleotide	621					637	640						
Amino Acid	208					213	214						
Sequence 1.	ATAGCGGGTAATGAG					CGT	AGT	ACTAACTTAATATCAAAC					
Translation		I	A	G	N	E	R	S	T	N	L	I	S	N
Sequence 2.	ATAGCGGGTAATGAG					TAT	CTT	ACTAACTTAATATCAAAC					
Translation		I	A	G	N	E	Y	L	T	N	L	I	S	N
Sequence 3.	ATAGCGGGTAATGAG					TAT	GAT	ACTAACTTAATATCAAAC					
Translation		I	A	G	N	E	Y	D	T	N	L	I	S	N
WT-CYP 119	ATAGCGGGTAATGAG					ACT	ACA	ACTAACTTAATATCAAAC					
Translation		I	A	G	N	E	T	T	T	N	L	I	S	N

3.2.3. Screening of CYP119 Thr213-Thr214 Mutant Library

After construction and confirmation of the mutant library, CYP119 mutants were screened for improved peroxidation activity. Fluorescence emission of resorufin created by mutants, after ten minutes of incubation at room temperature, were measured with 595 nm fluorescent filter that has 585 nm to 600 nm range on detecting fluorescent emission and compared to fluorescence emission created by WT CYP119. Horseradish peroxidase (HRP) in the reaction mixture (10 µM Amplex Red and 1 mM H_2O_2 in 50 µM pH 7.4 KPi

buffer) was used as positive control and cells with pET20b plasmid in the reaction mixture and well that contains only reaction mixture were used as the negative control. Then selected improved activity CYP119 mutants were grown on a bigger scale to confirm their improved activities and for further analysis.

Peroxidase activity assay with Amplex Red was performed on 68 mutants to determine improved activity. As shown in Figure 3.9. among 68 mutants there were nine mutants that showed improved activity; B1/16, B1/26, B2/15, B2/17, B2/22, B2/23, B2/25, B2/29, and B2/30. These mutant CYP119 enzymes were selected as further confirmation with 8ml expression test. 8ml expression test was done as follow, each mutant was grown in mL LB media with 100 µg/mL ampicillin for overnight at 37 °C and 220 RPM. Then, 30 µL of each grown culture were transferred to 8 mL of 2xYT media containing 100 µg/mL ampicillin. After reaching OD_{600} of 0.8 at 37 °C and 220 RPM, IPTG was added to each culture. Then cultures were incubated for 25 hours at 30 °C and 220 RPM. Proteins were extracted with SoluLyse and extracted proteins were subjected to Amplex Red Assay.

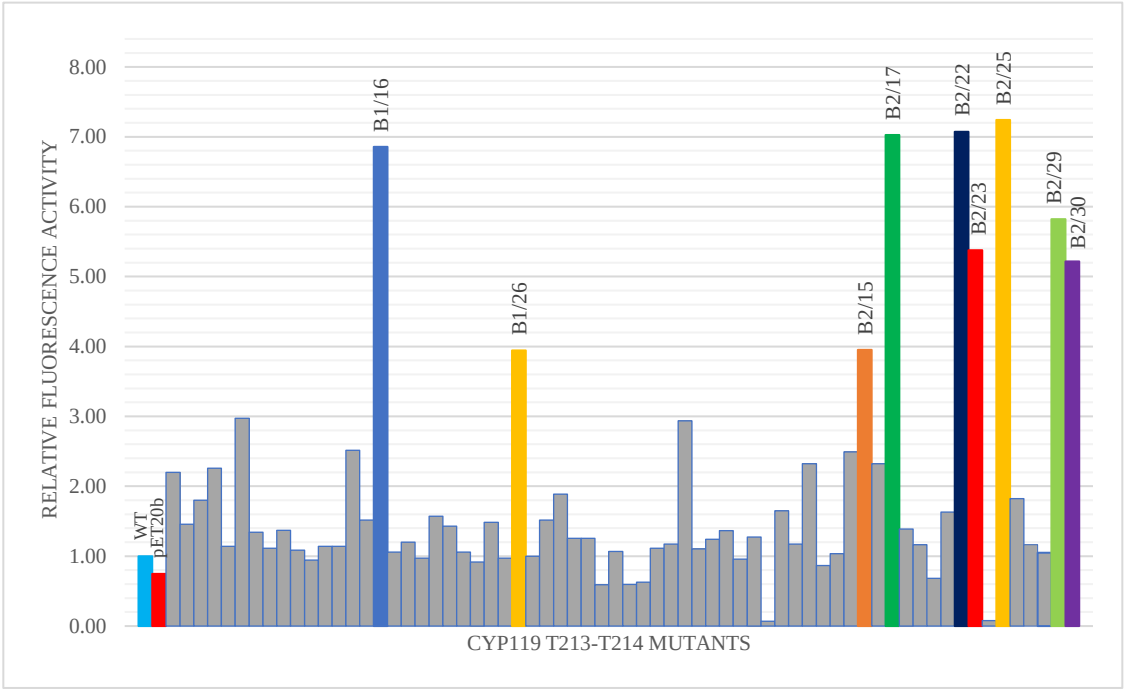


Figure 3.9. Fluorescence activities of Thr213-Thr 214 CYP119 mutants relative to WT CYP119. Enzymes were extracted with SoluLyse treatment.

Table 3.2. Improved CYP119 mutants.

Mutant	Increase in Activity	Mutant	Increase in Activity
B1/16	6.8-fold	B2/23	5.4-fold
B1/26	3.9-fold	B2/25	7.2-fold
B2/15	3.9-fold	B2/29	5.8-fold
B2/17	7-fold	B2/30	5.2-fold
B2/22	7-fold		

3.2.4. Confirmation of Improved Activity CYP119 Mutants

E. coli BL21 (DE3) bacteria that contain plasmid with improved activity mutants were grown in 8ml 2xYT media and proteins were extracted with SoluLyse. Amplex Red peroxidation activity assay was performed. Figure 3.10. shows activity test results done with selected improved activity CYP119 mutants. Activity increase quantities were shown in Table 3.3. Among nine mutants B1/16, B2/15, B2/17, B2/22, and B2/29 confirmed their improved peroxidase activity. Plasmids isolated from selected improved mutants were sequenced. Sequence analysis of the improved CYP119 mutants were shown in Table 3.4. B1/16 has T213D-T214N substitutions, B2/15 has T213C-T214D substitutions, B2/17 has T213D-T214I substitutions, B2/22 and B2/29 have the same substitutions T213R-T214Y. Coordination of the substituted amino acids relative to heme iron were shown in Figure 3.11.

Table 3.3. Confirmation of improved activity mutants

Mutant	Increase in Activity	Mutant	Increase in Activity
B1/16	1.1-fold	B2/23	1.6-fold
B2/15	2.6-fold	B2/25	1.6-fold
B2/17	1.7-fold	B2/29	1.8-fold
B2/22	1.9-fold	B2/30	1.4-fold

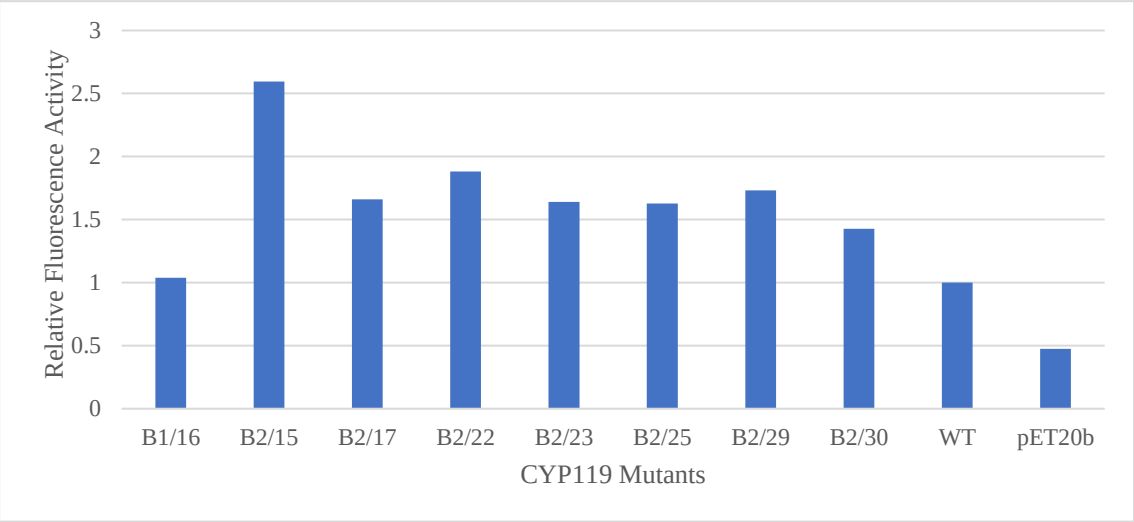


Figure 3.10. Confirmation of improved CYP119 Thr213-Thr214 mutant enzyme candidates, each bacterium was grown from bacterial glycerol stocks from the replica plate.

Table 3.4. Sequence analysis of plasmids from improved CYP119 mutant enzymes.

Nucleotide	621					637	640										
Amino Acid	208					213	214										
WT CYP119	ATAGCGGGTAATGAG					ACT	ACA	ACTAACTTAATATCAAAC									
Translation		I	A	G	N	E	T	T		T	N	L	I	S	N		
B1/16	ATAGCGGGTAATGAG					GAT	AAT	ACTAACTTAATATCAAAC									
Translation		I	A	G	N	E	D	N		T	N	L	I	S	N		
B2-15	ATAGCGGGTAATGAG					TGT	TGT	ACTAACTTAATATCAAAC									
Translation		I	A	G	N	E	C	C		T	N	L	I	S	N		
B2-17	ATAGCGGGTAATGAG					GAT	ATT	ACTAACTTAATATCAAAC									
Translation		I	A	G	N	E	D	I		T	N	L	I	S	N		
B2-22	ATAGCGGGTAATGAG					CGT	TAT	ACTAACTTAATATCAAAC									
Translation		I	A	G	N	E	R	Y		T	N	L	I	S	N		
B2-29	ATAGCGGGTAATGAG					CGT	TAT	ACTAACTTAATATCAAAC									
Translation		I	A	G	N	E	R	Y		T	N	L	I	S	N		

Molecular dockings of Amplex Red to WT CYP119 and improved CYP119 mutants was done by Ekin Kestevur in PyRosetta program with DockMCM protocol. As shown in Figure 3.12. Amplex Red reagent (substrate) has 6.7 Å distance to active site of WT CYP119 enzyme and there is no hydrogen bonding between substrate and enzyme's active site. Ten rounds of molecular docking of Amplex Red to WT CYP119 resulted -513 Rosetta Energy Unit (REU) docking score. Rosetta score is an alternative for free energy of the complexes. Therefore models with lower score indicate more favorable energies[57]. Additionally, hydrogen bonding (H-bonding) analysis was done on the dockings, and substrate has no H-bonding to enzymes active site.

B2/15 mutant with T213C-T214C substitutions has a docking score of -1199 REU which is better score than WT CYP119. Nitrogen atom of Amplex Red has 5.4 Å distance to heme iron of enzyme and substrate makes one H-bond with Thr257 residue in the active site as shown in Figure 3.13. Therefore, improved activity of B2/15 mutant enzyme can be due to increased affinity for Amplex Red.

B2/17 mutant with T213D-T214I substitutions resulted -1196 REU docking score that is lower than WT CYP119. Distance of nitrogen atom of Amplex Red to heme iron is 5.0 Å and substrate makes one H-bond to protoporphyrin ring of heme group as seen in Figure 3.14. Docking results imply improved activity of B2/17 mutant can be due to better binding of substrate.

B1/16 mutant with T213D-T214N substitutions gave a docking score of -1184 REU which indicate a better binding of Amplex Red to enzymes active site than WT CYP119. N atom of substrate has 6.4 Å to heme iron and substrate makes total of two H-bonds, one with Arg256 and one with Thr257 as shown in Figure 3.15 with green continuous lines. Consequently, B1/16 mutant exhibits improved activity toward Amplex Red.

B2/22 and B2/29 have exact same substitutions T213R-T214Y which results in -1184 REU docking score that leads to a more favorable energy. Nitrogen atom in of Amplex Red has 6.7 Å distance. This distance is the same with WT CYP119 however substrate makes one H-bond with Gly156 residue in the active site of T213R-T214Y mutant enzymes as shown in Figure 3.16. Consequently, having a better docking and score may explain the improved activity of T213R-T214Y mutant enzyme.

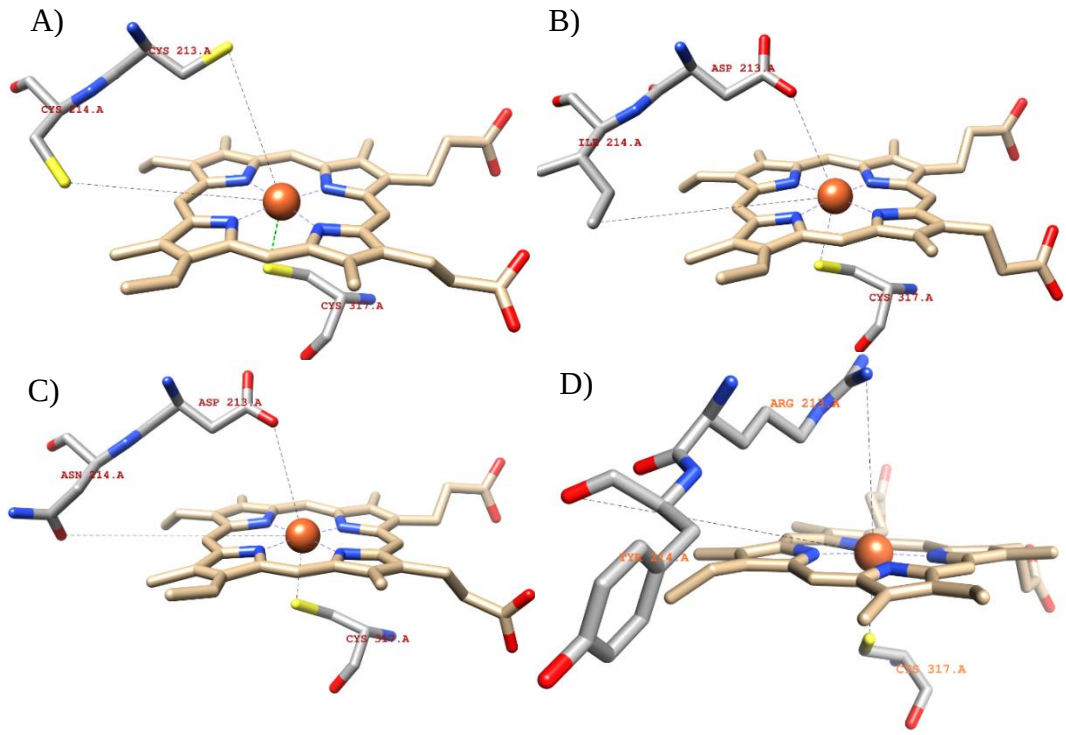


Figure 3.11. Improved activity CYP119 mutant enzymes. A) B2/15; T213C-T214C, B) B2/17; T213D-T214I, C) B1/16; T213D-214N, D) B2/22, B2/29; T213R-T214Y (both improved mutants have the same amino acid sequence)

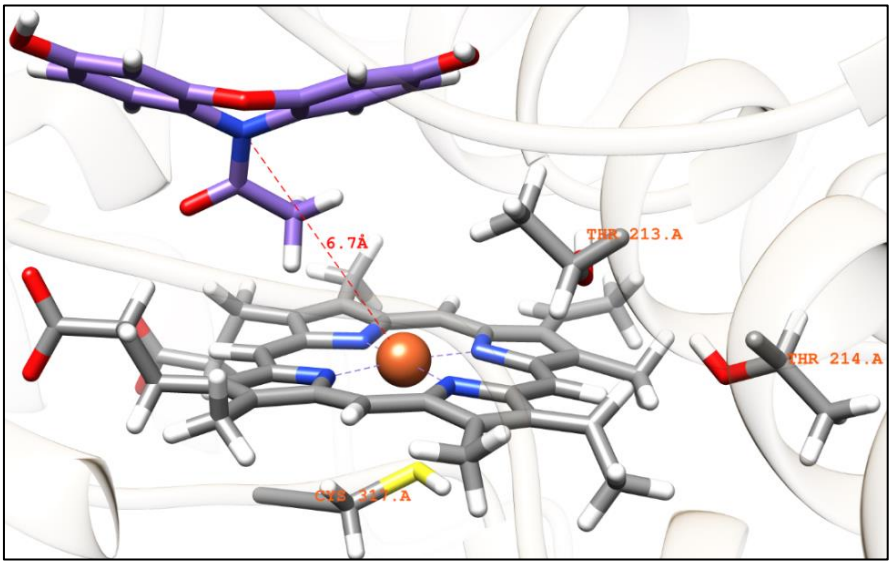


Figure 3.12. Molecular docking of WT CYP119 with Amplex Red. Purple reagent is Amplex Red, red dashed line represents the distance on nitrogen (N) atom of Amplex Red to heme iron

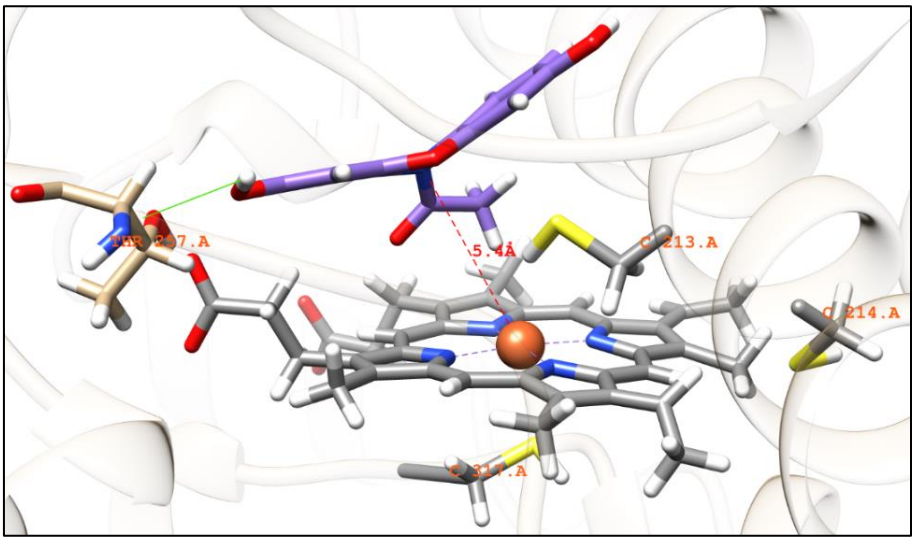


Figure 3.13. Molecular docking of B2/15 with T213C-T214C substitutions with Amplex Red. Purple reagent is Amplex Red, red dashed line represents the distance on nitrogen (N) atom of Amplex Red to heme iron. Green continuous lines represent the Hydrogen bonding between substrate and the active site residues.

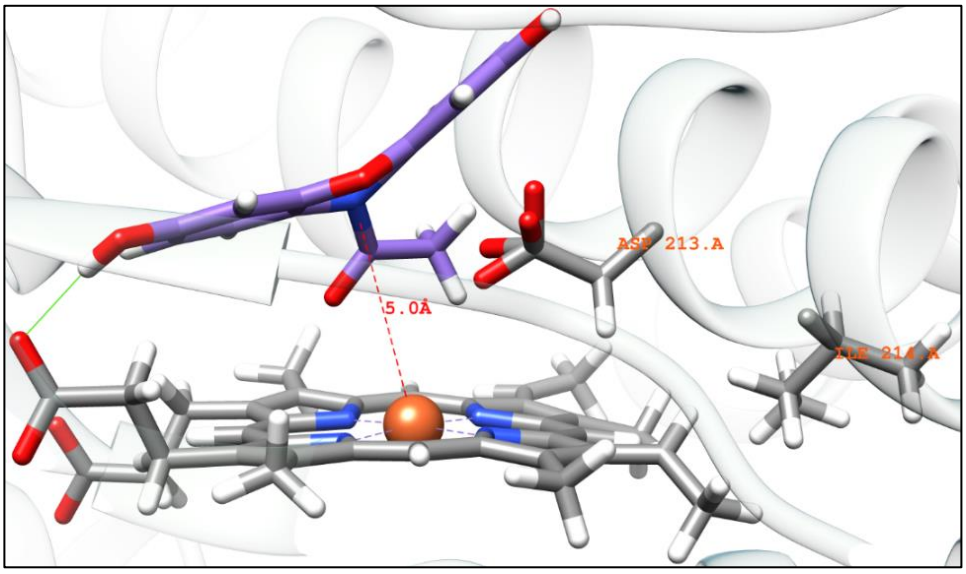


Figure 3.14. Molecular docking of B2/17 with T213D-T214I substitutions with Amplex Red. Purple reagent is Amplex Red, red dashed line represents the distance on nitrogen (N) atom of Amplex Red to heme iron. Green continuous lines represent the Hydrogen bonding between substrate and the active site residues.

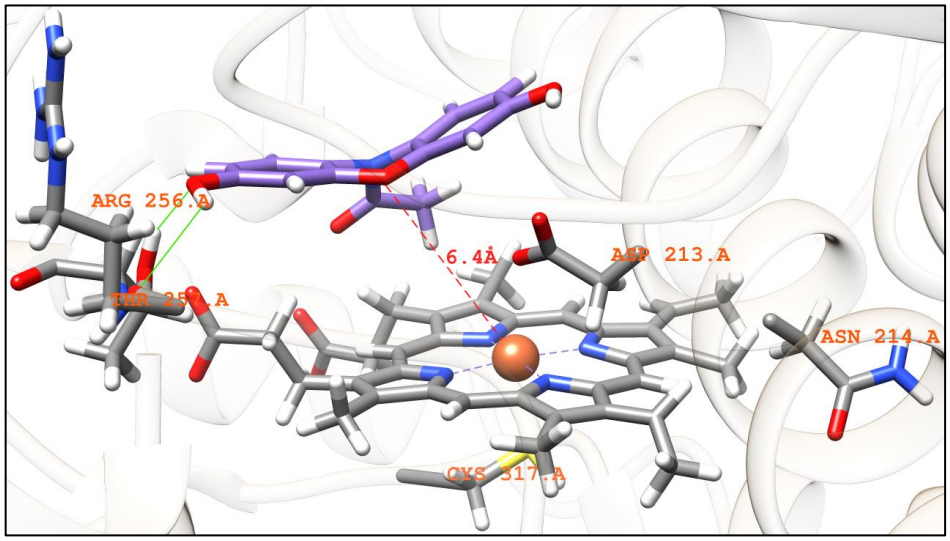


Figure 3.15. Molecular docking of B1/16 with T213D-T214N substitutions with Amplex Red. Purple reagent is Amplex Red, red dashed line represents the distance on nitrogen (N) atom of Amplex Red to heme iron. Green continuous lines represent the Hydrogen bonding between substrate and the active site residues

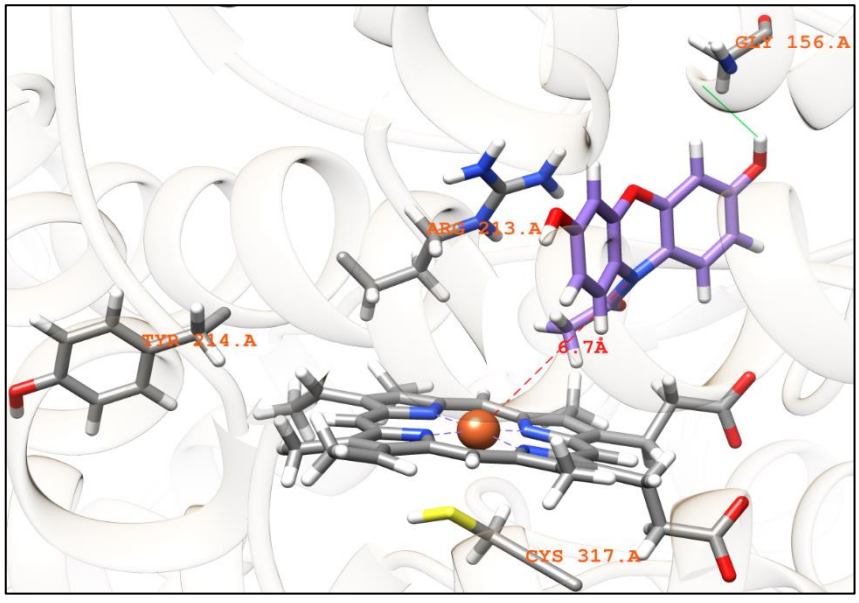


Figure 3.16. Molecular docking of B2/22 and B2/29 with T213R-T214Y substitutions with Amplex Red. Purple reagent is Amplex Red, red dashed line represents the distance on nitrogen (N) atom of Amplex Red to heme iron. Green continuous lines represent the Hydrogen bonding between substrate and the active site residues.

Table 3.5. Substitutions and scores of Amplex Red docking of WT CYP119 and improved CYP119 mutant enzymes.

Enzyme	Substitutions	Docking Score (REU)
WT CYP119	-	-513
B1/16	T213D-T214N	-1184
B2/15	T213C-T214C	-1199
B2/17	T213D-T214I	-1196
B2/22 & B2/29	T213R-T214Y	-1184

3.2.5. Spectroscopic Analysis of Improved Activity CYP119 Mutants

Optical absorption spectra of the WT CYP119 and improved CYP119 mutants were shown in Figure 3.17. WT CYP119 show a Soret band at 415 nm wavelength and improved CYP119 mutants have shifted Soret bands in 415 nm – 427 nm range. Mutations in the active site can change the Soret peaks. Soret peaks of WT CYP119 and selected CYP119 mutants were shown in Table 3.6. Shifts in Soret maximum absorbance can be a result of changes that occurred in the electronic properties of heme group of the enzymes as mutations were in the heme distal pocket.

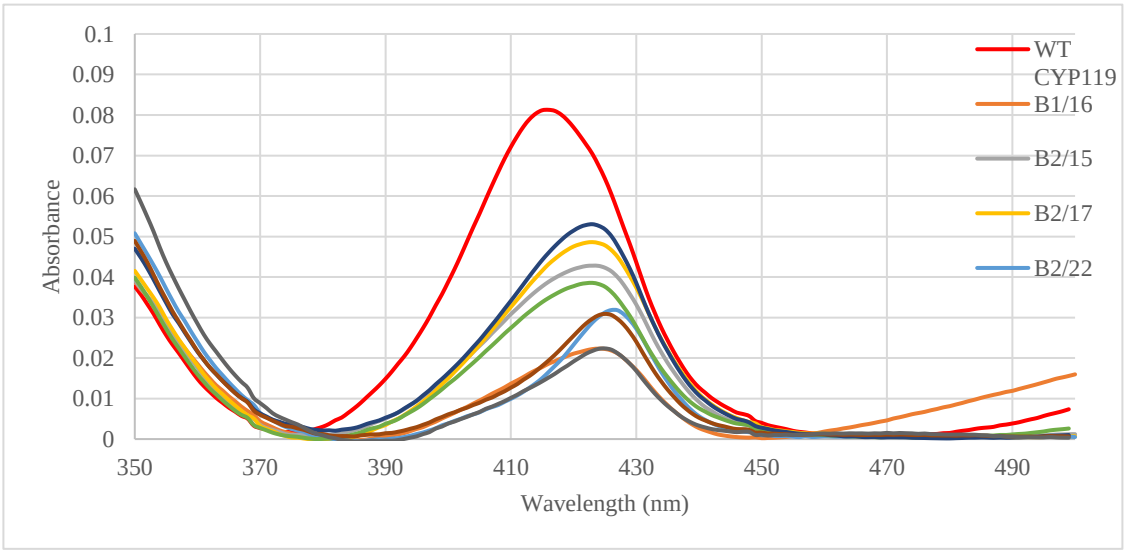


Figure 3.17. UV absorption spectra of WT CYP119 and improved CYP119 mutants.

Table 3.6. Wavelengths of Soret peaks observed in UV spectra of WT CYP119 and CYP119 mutants.

Enzyme	Soret Maximum (nm)	Enzyme	Soret Maximum (nm)
WT CYP119	416	B2/23	424
B1/16	424	B2/25	424
B2/15	423	B2/29	426
B2/17	423	B2/30	426
B2/22	426		

3.3. Mutations of CYP119 at Val151 and Phe153

Val151 and Phe153 residues are positioned close to the heme group in the active site of CYP119 enzyme and they are spatially close to each other. Systematic mutations at these sites by applying CAST method might result in an increase in the peroxidation activity of CYP119.

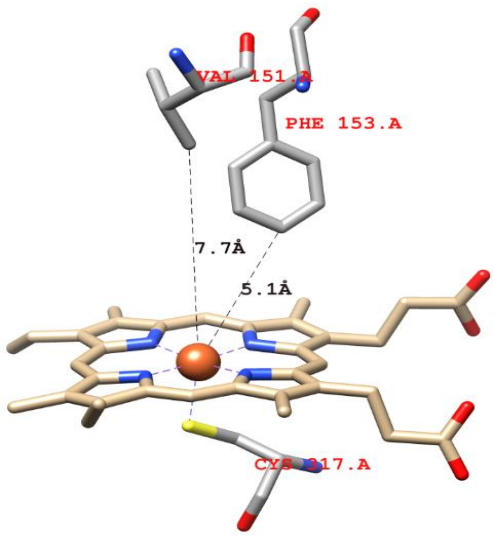


Figure 3.18. Distances of Val151-Phe153 residues to heme iron.

3.3.1. Creation of Mutant Genes by Polymerase Chain Reaction

CAST method was applied to create mutant genes. PCR was done with degenerate primer mix to cause changes at Val151 and Phe153 residues in WT CYP119 gene in pET11a. PCR product was treated with KLD enzyme mix to acquire only mutant genes in the PCR product. PCR run was confirmed by running PCR product on %1 agarose gel. Length of CYP119 in pET11a is 6748bp and accomplishment of PCR run was confirmed in Figure 3.19. Bands in the agarose gel image confirm that the PCR was successfully done.

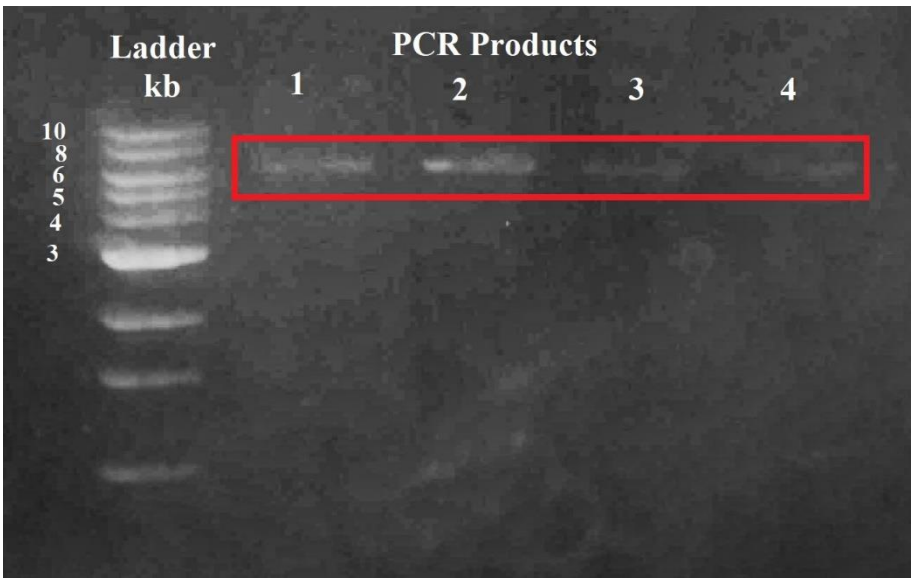


Figure 3.19. Agarose gel image of KLD treated PCR products, length of PCR product (CYP119 in pET11a) is 6.7kb. PCR product bands were shown with the rectangle.

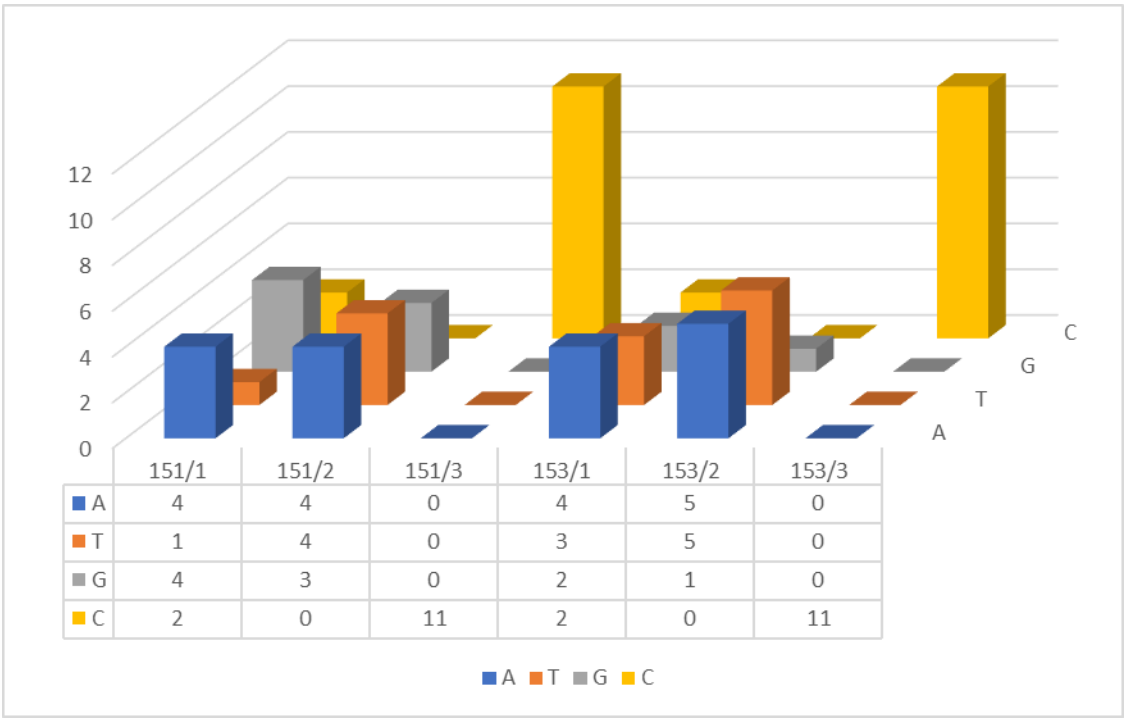
3.3.2. Construction of the CYP119 Val151 – Phe153 Mutant Library

Mutant library construction was completed by transforming mutant genes created in 3.3.1 obtained to high-efficiency competent *E. coli* BL21 (DE3) cells. Transformation

was done by heat-shock transformation. 50 µL competent *E. coli* BL21DE3 cells were mixed with 5 µL of the mutant genes and incubated on ice for 30 mins. After incubation cells were treated with heat shock at 42 °C for 45 seconds followed by incubation on ice for 2 mins. The 950 µL of SOC media was added to cells and incubated for 1 hour at 37 °C and 220 RPM.

After transformation bacteria were grown on LB agar plate and three random colonies were picked from plates for confirmation of mutations. Sequence analysis of plasmids from eleven random colonies confirmed that mutations occurred at Val151 and Phe153 residues as shown in Table 3.7.

Frequencies of substituted amino acids were analyzed in order to determine possible mutational bias. Sequence analysis indicated that created mutant library is diverse and has no mutational bias present as shown in Figure 3.20. Furthermore, when 11 sequences from different colonies were examined, there are eight different amino acids employed out of twelve possible amino acids at the 151st position, and when sequences at the153rd position was examined there are six different amino acids employed, out of twelve possible amino acids, by using NDT codons, Table 3.7. shows the sequences of plasmids from randomly selected eight colonies.



	151/1	151/2	151/3	153/1	153/2	153/3
A	4	4	0	4	5	0
T	1	4	0	3	5	0
G	4	3	0	2	1	0
C	2	0	11	2	0	11

Figure 3.20. Frequencies of substituted bases in the NDT codons encoding amino acids at 151st and 153rd position.

Table 3.7. Sequence analysis of plasmids isolated from randomly selected colonies.

Nucleotide	338	351	359
Amino Acid	147	151	153
WT CYP119	TGGTCAGACTTA	GTC GCA TTC	AGGTTGGGTAAG
Translation	W S D L	V A F	R L G K
Sequence 1	TGGTCAGACTTA	ATT GCA CTT	AGGTTGGGTAAG
Translation	W S D L	I A L	R L G K
Sequence 2	TGGTCAGACTTA	CTT GCA TTT	AGGTTGGGTAAG
Translation	W S D L	L A F	R L G K
Sequence 3	TGGTCAGACTTA	TTT GCA AAT	AGGTTGGGTAAG
Translation	W S D L	F A N	R L G K
Sequence 4	TGGTCAGACTTA	AAT GCA TAT	AGGTTGGGTAAG
Translation	W S D L	N A Y	R L G K
Sequence 5	TGGTCAGACTTA	CAT GCA GTT	AGGTTGGGTAAG
Translation	W S D L	H A V	R L G K
Sequence 6	TGGTCAGACTTA	AGT GCA GGT	AGGTTGGGTAAG
Translation	W S D L	S A G	R L G K
Sequence 7	TGGTCAGACTTA	GTT GCA AAT	AGGTTGGGTAAG
Translation	W S D L	V A N	R L G K
Sequence 8	TGGTCAGACTTA	GGT GCA TAT	AGGTTGGGTAAG
Translation	W S D L	G A Y	R L G K
Sequence 9	TGGTCAGACTTA	GAT GCA AAT	AGGTTGGGTAAG
Translation	W S D L	D A N	R L G K
Sequence 10	TGGTCAGACTTA	AAT GCA CTT	AGGTTGGGTAAG
Translation	W S D L	N A L	R L G K
Sequence 11	TGGTCAGACTTA	GGT GCA ATT	AGGTTGGGTAAG
Translation	W S D L	G A I	R L G K

3.3.3. Screening of CYP119 Val151-Phe153 Mutant Library

CYP119 mutants were screened for improved peroxidation activity. Fluorescence emission of resorufin created by mutants, after ten minutes of incubation at room temperature, were measured with 595 nm fluorescent filter that has 585 nm to 600 nm range on detecting fluorescent emission and compared to fluorescence emission created by WT CYP119. Horseradish peroxidase (HRP) in the reaction mixture (10 µM Amplex Red and 1 mM H_2O_2 in 50 µM pH 7.4 KPi buffer) was used as positive control and cells with pET20b plasmid in the reaction mixture and well that contains only reaction mixture were used as the negative control. Then selected improved activity CYP119 mutants were grown on a bigger scale to confirm their improved activities and for further analysis.

Additionally, UV absorbance spectra between 300 and 700 nm of C10 coded proteins were measured because proteins extracted from pET20b plasmid containing bacteria were exhibiting high fluorescent values while performing the activity assay on proteins even though, protein extraction from pET20b containing bacteria does not turn Amplex Red into resorufin as seen in Figure 3.22. and Figure 3.22. Also, protein extractions from pET20b does not have any contaminations from peroxidase activity exhibiting cultures because no Soret peaks are present at 415 nm as shown in Figure 3.22. and no peaks are present at the 570 nm as seen in Figure 3.21. which is the wavelength of resorufin's. As an additional negative control, pUC19 plasmid containing bacteria were grown and proteins from the culture were extracted. Figure 3.21. and Figure 3.22. also indicates, WT CYP119 enzyme and mutant CYP119 enzymes contain heme since Soret peaks are present at 415 nm wavelength and have peaks at 570 nm that shows resorufin presence. Negative control, pET20b containing bacteria, was replaced with pUC19 containing bacteria. There were several tests done on protein extracts from pET20b containing bacteria such as heat treatment at 60 °C for 20 mins, increasing the centrifugation time from 10 mins to 30 mins in order to investigate if there are any interference from cell debris. However, the reason that protein extraction from pET20b containing bacteria exhibiting high values in fluorometric Amplex Red assay is currently unknown.

Peroxidase activity assay with Amplex Red was performed on 124 mutants to determine improved activity. As shown in Figure 3.23. among 124 mutants there was

only one mutant that showed improved activity; C7/11. This mutant CYP119 enzyme was selected as further confirmation with 8 mL expression test (Section 3.2.3).

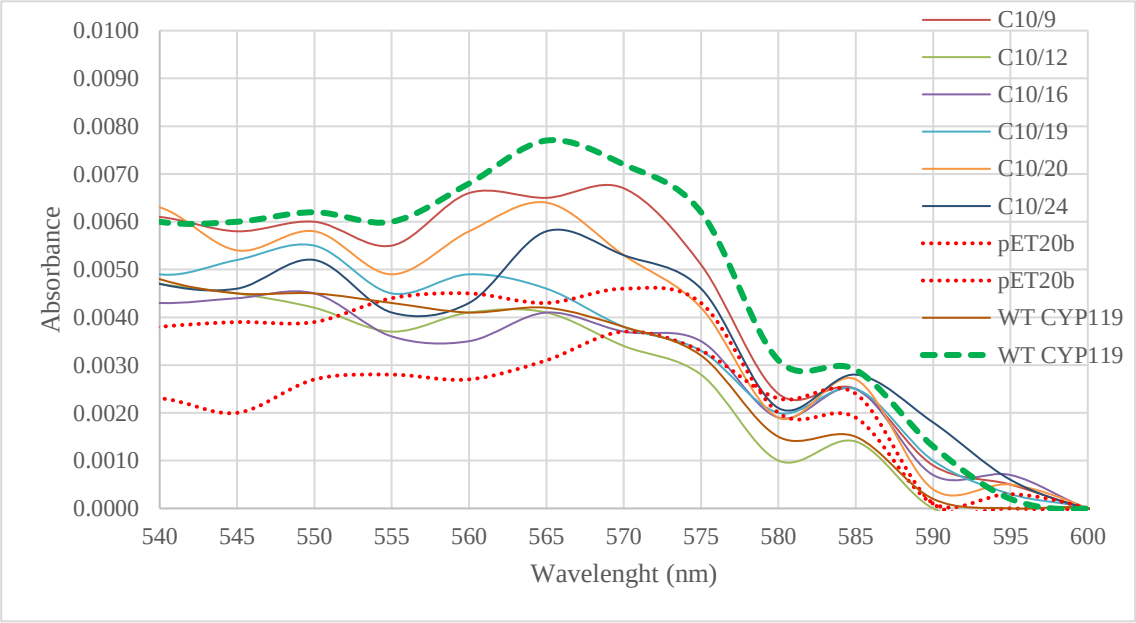


Figure 3.21. UV absorbance spectra of WT CYP119, pET20b and C10 series Val151-Phe153 mutant CYP119 enzymes showing resorufin content at 585 nm.

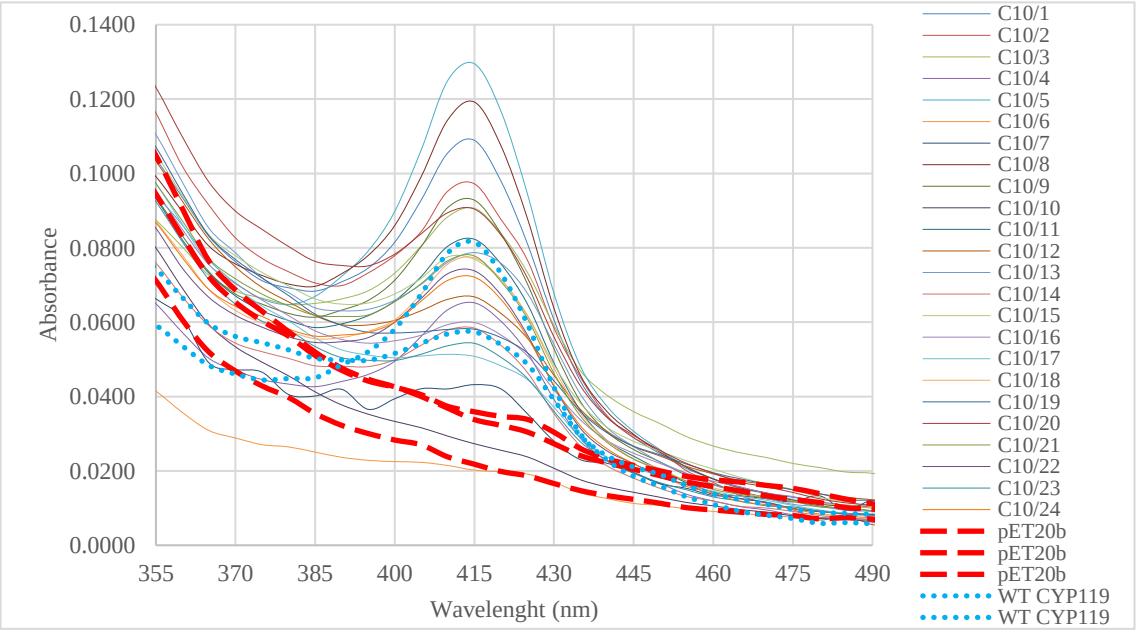


Figure 3.22. UV absorption spectra of WT CYP119 and improved CYP119 mutants.

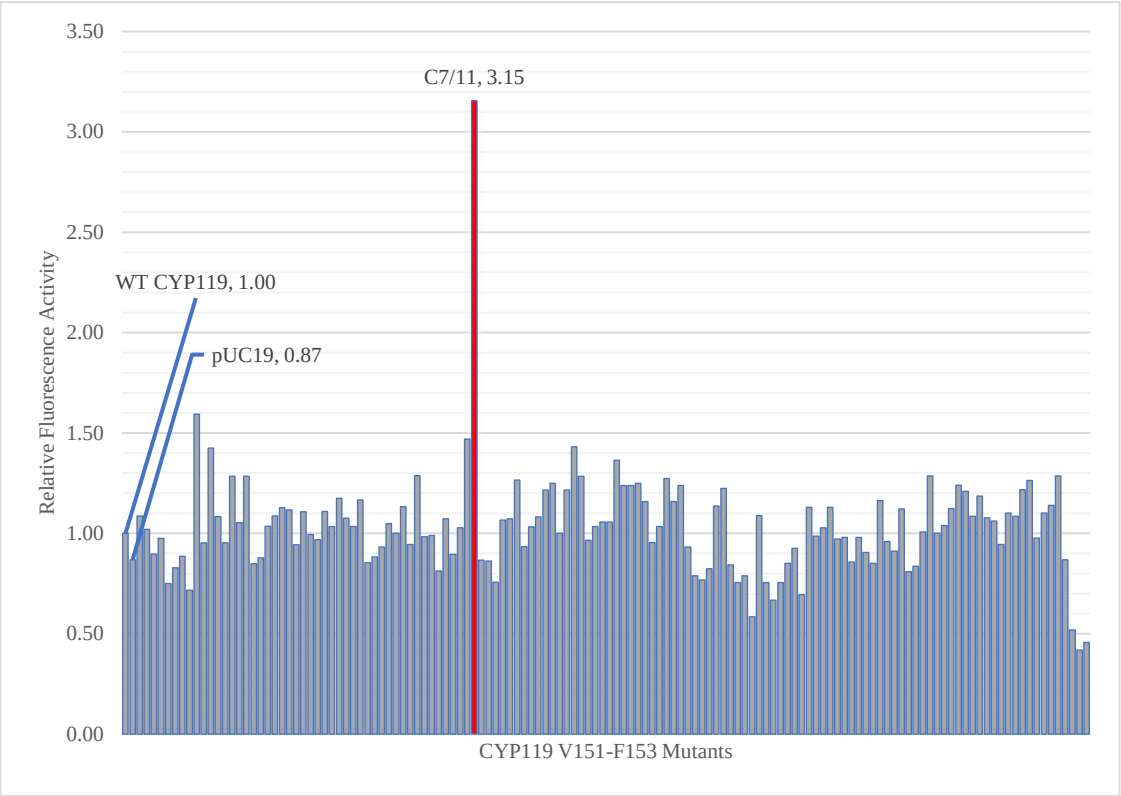


Figure 3.23. Fluorescence activities of Val151-Phe153 CYP119 mutants relative to WT CYP119. Enzymes were extracted with SoluLyse treatment.

3.3.4. Confirmation of Improved Activity CYP119 Mutants

E. coli BL21 (DE3) bacteria that contain plasmid with improved activity mutants were grown in 8ml 2xYT media and proteins were extracted with SoluLyse. Amplex Red peroxidation activity assay was performed. Figure 3.24 indicates that C7/11 mutant thatcontains substituted amino acids at Val151-Phe153 positions does not have improved peroxidation activity toward Amplex Red.

Confirmation test that resulted in similar fluorescence activity with WT CYP119, indicates Val151-Phe153 amino acid pair is not catalytically significant and thus does not have any effect on the peroxidation activity of CYP119 on Amplex Red.

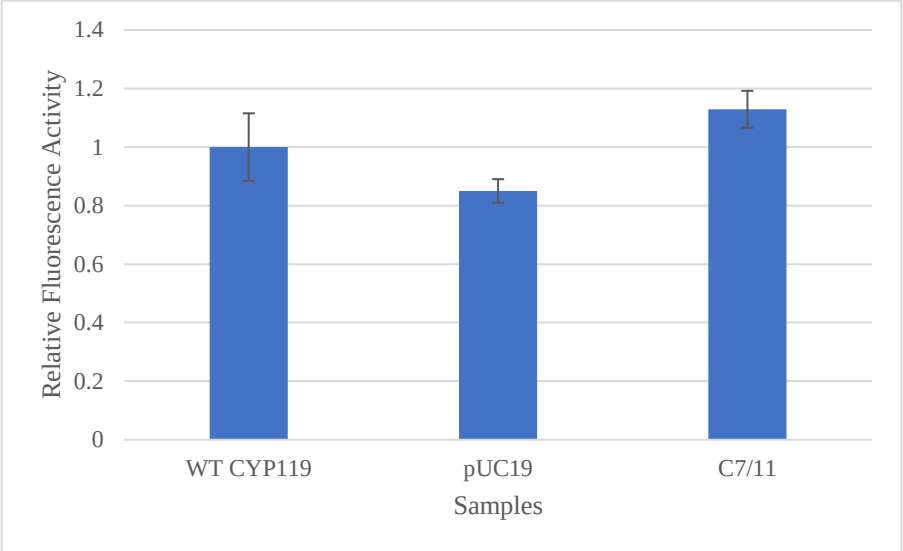


Figure 3.24. Confirmation of improved activity candidate C7/11.

3.3.5. Determination of Extracted Protein Concentrations

Concentrations of the SoluLyse extracted proteins were determined by using Bradford assay. Formula derived from standard curve was used to calculate the concentrations of the extracted proteins. As shown in Table 3.8. concentrations of extracted mutants were close to WT CYP119 extract protein concentrations, therefore the changes in activity in the assay were not due to difference in cell lysis.

Table 3.8. Concentrations of extracted proteins from WT CYP119 and mutant enzymes

Enzyme	Concentration (mg/mL)	Enzyme	Concentration(mg/mL)
WT CYP119	3.47	C4/6	4.13
WT CYP119	3.21	C4/7	5.62
B1/16	5.36	C4/8	4.50
B2/15	4.69	C5/6	2.73
B2/22	4.35	C5/7	2.23
C2/4	2.61	C5/8	2.27
C2/7	2.13		

CHAPTER 4

CONCLUSION

Developments in the recombinant DNA technology have significant contributions to protein engineering studies. Biocatalysts have significant advantages over chemical catalysts such as; high substrate specificity, enantioselectivity, chemoselectivity and most significantly being environmentally friendly. Such properties can be improved to obtain biocatalysts with desired properties. Thus, engineered biocatalysts can replace chemical catalysts used in industrial applications. In this study, CYP119, a member of P450 protein family was engineered in order to improve its peroxidation activity by using H_2O_2 as an oxidant. Two different mutant libraries of CYP119 that has substituted amino acids at Thr213-Thr214 and Val151-Phe153 positions were created by applying CASTing method and these mutant libraries were screened for their improved peroxidation activity toward Amplex Red. Several optimizations were done on the Amplex Red assay such as, selection of blank and addition of EDTA to Amplex Red mixture. Additionally, several tests regarding sensitivity of the fluorometric measurement device, effect of incubation time and effect of freezing and thawing cycles on enzyme activity were conducted on the Amplex Red assay. In Thr213-Thr214 mutant library screening, a total of five improved mutants out of 68 mutants were determined; B1/16 (T213D-T214N), B2/15 T213C-T214C), B2/17 (T213D-T214I), B2/22 (T213R-T214Y) and B2/29 (T213R-T214Y). Among improved mutants, B2/22 and B2/29 shares the same mutations at determined sites 213 and 214. Thus, four different improved mutants were determined. Docking studies with improved mutants imply that activity Thr213-Thr214 CYP119 mutations facilitates the binding of substrate Amplex Red to active site of the enzyme thus enhancing the peroxidation of Amplex Red into resorufin. In Val151-Phe153 mutant library screening no improved activity mutant was determined. This indicated amino acid substitutions at Val151-Phe153 has no significant effect on the peroxidation activity of CYP119 enzyme.

Consequently, this study enlightened effects of substituted amino acid substitutions at positions, 213-214 and 151-153 of CYP119 as well as an optimized and efficient screening method. This will contribute to enzyme engineering studies for creating enzymes with desired properties for industrial purposes.

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