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Temperature Dependence on Enzymatic Activity of A82F F87A Mutant and Wild-type Cytochrome P450 BM-3

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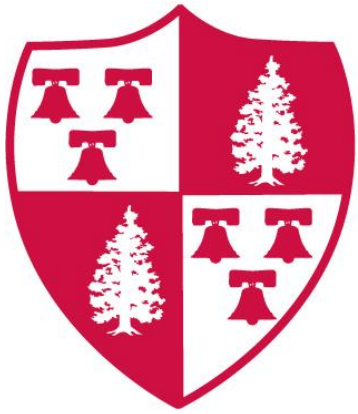

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Abstract

Cytochrome P450 is an enzyme mainly found in liver cells. It plays a key role in the breakdown of drugs and toxins by hydroxylating substrates making the substrates more water soluble. P450s are also involved in cholesterol and hormone synthesis. The third P450 from bacterium Bacillus Megaterium (BM3) is used as a model P450 enzyme due to its structural similarity to human P450s and due to its efficiency in the hydroxylation of fatty acids. Within the enzyme's active site, substrates can occupy a distal or proximal conformation monitored by UV-vis spectroscopy. The proximal conformation is more effective when it comes to hydroxylation so a double mutant (A82F/F87A) was developed and it was hypothesized that the mutation would allow for the proximal conformation to be more prevalent and increase the catalytic efficiency. Enzymatic Studies with palmitic (C16:0), eicosanoic (C20:0), and arachidonic (C20:4) acids as substrates were performed for the wild-type and mutant enzyme. Michaelis–Menten Kinetics were used to determine Km, kcat, and catalytic efficiency of the enzymes. The results of the experiments have not supported the hypothesis and the wild-type enzyme has shown to be a more efficient enzyme than the mutant. However, an overall increase in the catalytic efficiency of both mutant and wild type was noted with increasing temperature. Future studies include further experimentation with different chain length and saturation levels of fatty acids as well as looking at the products of enzymatic reactions as a function of temperature by LC/MS

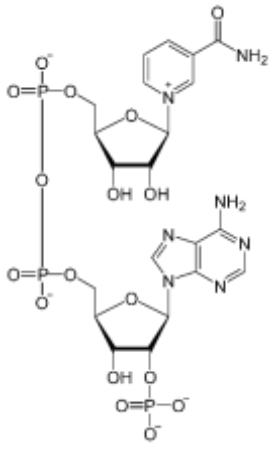
Introduction

Enzymes are proteins that exhibit a significant role in various cellular functions by catalytically speeding up chemical reactions, while remaining conserved. This characteristic is shown to be useful because it allows enzymes to be reused for numerous reactions. Enzymes speed up chemical reactions by binding to the substrate and lowering the activation energy required to initiate a reaction by stabilizing the transition state. Cytochrome P450s (P450s) are a class of enzymes that are found in all organisms. These enzymes are able to perform stereospecific oxidation of substrates through oxidation-reduction reactions. Moreover, P450s are responsible for the biosynthesis of hormones and cholesterol and for the metabolism of xenobiotics, drugs, toxins, vitamin D, and many other mammalian drugs. P450 is a heme-containing protein, meaning it has an iron porphyrin ring in its active site that allows for the transfer of electrons and the binding of oxygen. The heme lies at the end of a hydrophobic channel which attracts substrates via hydrophobic effect. This enzyme is an induced-fit enzyme, which contributes to its great catalytic qualities, since it uses conformation change for regulation. Upon binding of substrate, there is a protein conformational change, which signals the beginning of the P450 catalytic cycle.

Various bacterial and mammalian cytochrome P450 structures have been extensively studied. Bacterial P450s were first more heavily studied due to their water solubility, expression practicality, and the ease of purifying and crystalizing them. These bacterial P450s possess exceedingly high catalytic activities amongst their family of heme-containing monooxygenase enzymes. Different P450 crystal structures have been carefully studied in order to better understand the catalytic reactions that occur between the substrate and enzyme. A few essential amino acids were found to be conserved in the sequence of the numerous P450s studied due to their catalytic and structural importance to the enzyme's function. Similar substrate selectivity

was distinguished between P450s with similar amino acid sequences, however, an immense variation in amino acid sequence and thus substrate selectivity still exists. The third P450 from the bacterium, *Bacillus megaterium*, or P450 BM3 or CYP102A1 has one of the highest known monoxygenase catalytic activities amongst its family of P450s due to its efficient electron transfer from its cofactor, Nicotinamide adenine dinucleotide phosphate (NADPH) (*1*). This 119 kDa prokaryotic P450 model is unique because it receives its electrons during the redox reaction from an attached eukaryotic partner (*1*). The P450 BM3 fatty acid hydroxylase model is also thought of as an ideal model in studies due to its strong similarity to eukaryotic fatty acid hydroxylase and it can be expressed at high levels in *E. coli* (*1*). Moreover, this model is also similar to mammalian models in that it contains a P450 domain and a diflavin NADPH-cytochrome P450 reductase domain which further indicates that it is a good system to use for *in vitro* studies (*2*). It also plays a significant role in the detoxification of polyunsaturated fatty acids, such as arachidonic acid. The downside of this prokaryotic model, however, is that it is a mesothermophilic organism and so it grows at an optimal temperature of around 30 °C, thus it lacks the thermostability often demanded by the mammalian model it is representing (*3*). Nevertheless, it is still considered stable relative to human P450s (*1*).

NADPH is a substantial cofactor essential to various metabolic reactions. It serves as an excellent electron donner, or electron acceptor in its $NADP^+$ form, during oxidation-reduction reactions, such as those catalyzed by P450 enzymes. This molecule absorbs ultraviolet-visible light at a wavelength of 340 nm. The chemical redox reaction carried out by P450 would not be possible without NADPH as its cofactor. The redox reaction catalyzed by P450 is:



NADPH

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + NADP^+ + H_2O$$

where R is the substrate. As illustrated in the reaction above, during a cytochrome P450 catalyzed reaction, NADPH is oxidized to $NADP^+$ and the substrate is hydroxylated. As previously mentioned, the hydrophobic active site of P450 attracts molecular oxygen, and is therefore present as a reactant during a P450 chemical reaction. Protons from the surroundings also take part at the initiation of the reaction, allowing for a successful hydroxylation of the substrate, causing the substrate to become more water soluble.

The original P450 catalytic cycle was first proposed in 1971 by Estabrook *et al.* and it remains to be the most widely accepted mechanism for P450s (*4*). This proposed mechanism begins with the enzyme (P450) being at its resting state, which means there is a water molecule weakly bound to the sixth coordinate on the low spin Fe(III). Then, when the ligand is introduced, the water is displaced, creating a high spin state Fe(III) that allows the ligand to bind. Then the most likely pathway suggests that the first electron is added, reducing the high spin Fe(III) to high spin Fe(II). Molecular oxygen then binds to the reduced high spin Fe(II) and forms an unstable ferric superoxy complex. A second electron is then added to form a low spin peroxo iron complex. A proton is then added which forms a hydroperoxy iron complex and then the addition of a second proton removes the water molecule and results in a reactive ferryl (II) cation radical that is postulated to be responsible for oxidizing the substrate (*1*). The ligand is thus bound to the oxygen-iron complex and the product is formed, which is then released to allow the enzyme to return to its resting state, as seen in Figure 1.

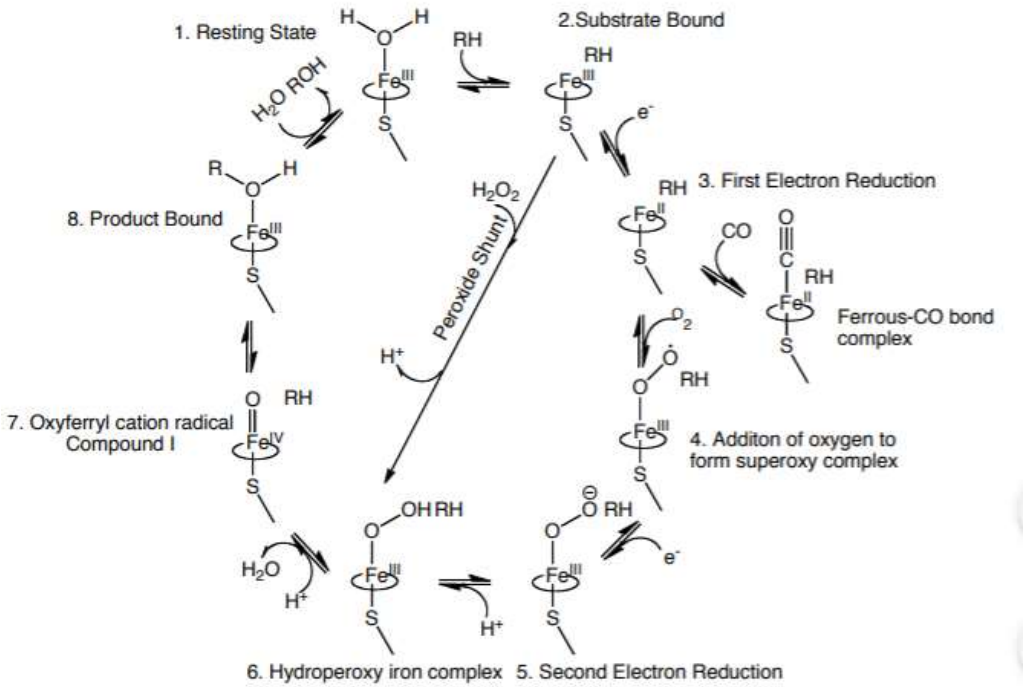
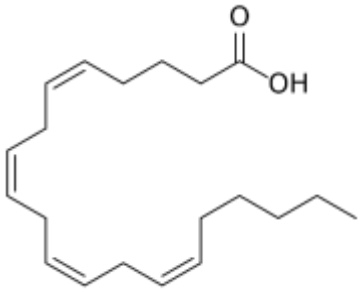


Figure 1. P450 proposed catalytic cycle

Possible substrates of P450 that take part in such a metabolic reaction include steroids, xenobiotics and most importantly emphasized in this study, fatty acids. Fatty acids are important building blocks of most complex lipids and are major components of cell membranes. They serve many functions in living organisms. Their long chain of carbons composed of numerous C-H bonds allow them to store large amounts of energy that the body can use for fuel. Fatty acids are classified based on the number of carbons in their carbon chains and the number of double bonds. The carbon chain length of fatty acids may range from 2 to 80 carbons; however, the most common fatty acids have carbon chain lengths that range from 12 to 24 carbons. Some common fatty acids that are a necessary component of the human diet include saturated fatty acids such as arachidic acid (C20:0), stearic acid (C18:0), palmitic acid (C16:0), and lauric acid (C12:0) and unsaturated fatty acids such as linoleic acid (C18:2) and arachidonic acid (C20:4). Fatty acids have shown to be ideal substrates to the cytochrome P450 enzyme, as well as the ideal substrate for the hydroxylation reaction by the P450 BM3 prokaryotic model.

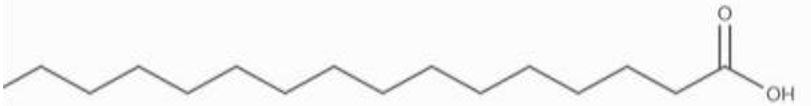
Arachidonic acid is a polyunsaturated fatty acid that consists of a twenty carbon-chain length with four cis-double bonds, or sites of unsaturation. Its structure indicates flexibility for protein interaction due to the hairpin conformation that results from the configurational isomerism of the fatty acid's unsaturation. In addition, under various temperatures, arachidonic acid is able to maintain membrane fluidity and continued interaction with molecular oxygen through various reactions due to its unique structure (*5*). This essential fatty acid is commonly found in all mammalian plasma membranes bound to phospholipids and it is also found in the muscles, liver, and brain. It is a regulator in pain and inflammatory responses in mammals, thus, emphasizing its significance. The natural substrate for P450s is not known, but previous studies using the P450 BM3 model with arachidonic acid as the ligand showed that BM3 was able to catalyze the oxidation of arachidonic acid with a rate constant larger than 17,000 min^{-1}, thus indicating that it is a favorable substrate with this model (*3*). Similarly, arachidic or eicosanoic acid is similar to but lacks the unsaturation of arachidonic acid. It consists of a straight twenty-carbon chain, and thus does not exhibit a hairpin conformation. Considering both fatty acids possess the same carbon-chain length, there is interest in the role saturation plays in hydroxylation reactions coupled with P450s.



Arachidonic Acid (C20:4)

Arachidic Acid (C20:0)

Palmitic acid is a saturated fatty acid consisting of a sixteen-carbon backbone. This particular fatty acid may be synthesized in the body via *de novo* lipogenesis or consumed dietarily (*6*). It makes up 20-30% of fatty acid content in the human body (*6*). Palmitic acid is linked to membrane proteins and it is the shortest available fatty acid present in animals. Its carbon-chain length and critical role in mammalian endogenous reactions makes it a fit ligand for studying hydroxylation reactions carried out by P450s.



Palmitic Acid (C16:0)

P450s are found in all organisms and play an important role in life processes. They hold critical roles in many of the reactions that occur in living organism, thus, they are targets for mutagenesis in laboratories to better understand the role and function of a particular amino acid. Knowledge of substrate-protein interactions are essential in designing better drug targets and for bioengineering. These membrane-bound hemeproteins possess complex structures with various functions. During the P450 catalyzed reaction of the hydroxylation of the substrates, two observed conformational dynamics of the substrate are at equilibrium: a distal and a proximal conformation (*7*). Previous studies suggest that to improve the catalytic activity of the enzyme, the substrate must be in the correct position for chemistry (*2*). It is hypothesized that the substrate needs to be in the proximal position for chemistry to occur. With the goal of shifting the conformational equilibrium towards the more catalytical active conformation, the proximal conformation, site-directed mutagenesis at two positions was achieved.

Extensive analysis of the P450 active site in the crystal structure with the substrate bound in the distal conformation shows a phenylalanine (F87) blocking the heme in the porphyrin ring

that is impacting the chemistry of the enzyme and the tail of a fatty acid in a hydrophobic pocket near alanine 82 (*8*). Phenylalanine is a hydrophobic, aromatic, nonpolar bulky amino acid and alanine is a small hydrophobic, nonpolar amino acid. During the distal ligand conformation, these two amino acids that are close in sequence, create a hydrophobic pocket near the active site that attracts hydrophobic substrates to it, which is proposed to decrease the catalytic activity of the enzyme and reduce the chemistry between enzyme and substrate. Fatty acids, which are again the substrates used in this study, have hydrophobic tails consisting of hydrocarbon chains that are attracted to this hydrophobic pocket. As a result, the fatty acid tails move towards the hydrophobic pocket rather than the active site which is unfavorable. A double point mutation was designed to achieve greater enzymatic chemistry by switching the spots of the alanine and phenylalanine. The mutant, A82F F87A, was hypothesized to increase the catalytic efficiency of the enzyme by disturbing the equilibrium observed in wild-type P450 kinetic studies through guiding the equilibrium towards an increase in the proximal ligand confirmation. It was also hypothesized that the movement of the substrate towards the correct position for catalysis is temperature dependent. Figure 2 illustrates the distal ligand conformation seen by x-ray crystallography and the proposed proximal conformation constructed with N-palmitoylglycine as the substrate by McDermott *et al.* by MD simulations (*9*).

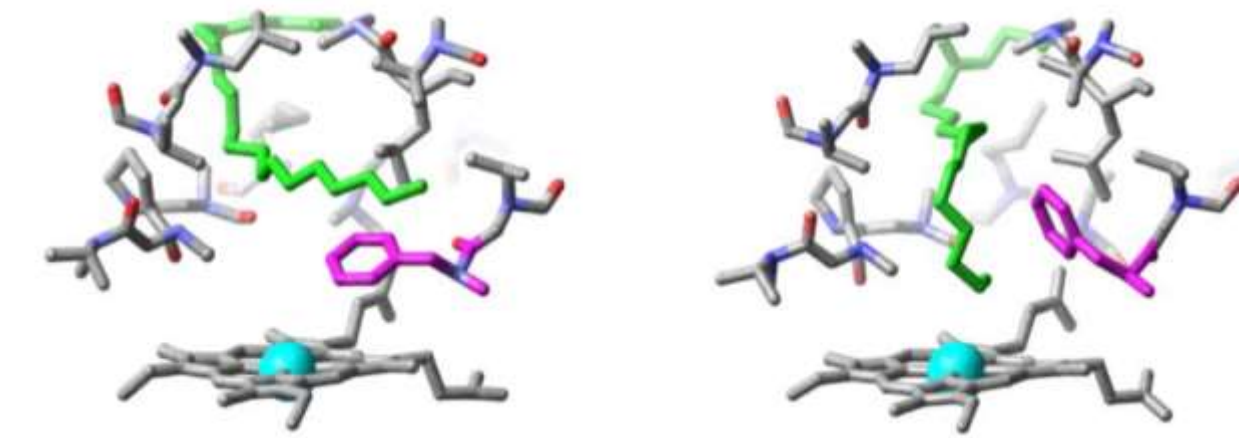


Figure 2. The crystal structure of the distal ligand conformation (left) of cytochrome P450 BM3 with N-palmitoylglycine (NPG) and the proximal ligand conformation (right). The hydrophobic tail of NPG (green) is farther away from the heme iron (blue) in the distal conformation and it is suggested that F87 (purple) contributes to such an occurrence.

Prior spin state studies suggest that the resting state of the enzyme is low spin Fe(III) heme and that the distal sixth coordinating ligand is water, while the other five ligands consist of four nitrogen groups in the porphyrin ring and one sulfur from a cysteine residue (*9*). The cysteine residue bound to the heme-iron is essential for the monooxygenase activity of P450 (*3*). The optical absorption spectrum for the low spin state is observed at 418 nm where the enzyme dynamics indicate the presence of a distal ligand conformation in the presence of ligand. When the ligand, the fatty acid, is bound in the proximal conformation, the peak shifts to 390 nm corresponding to the shift from low spin state to high spin state. McDermott *et al.* suggested that the spin state conversion is temperature dependent (*9*). Thus, our goal is to conduct the effect of the mutation on the spin state and catalytic efficiency as a function of temperature.

Materials and Methods

All materials needed to carry out the various experiments were performed in the research lab of Dr. Jaclyn Catalano. Wild-type Cytochrome P450 BM3 and mutant A82F F87A were expressed and purified from previously published protocols as well as protocols retrieved from Dr. Catalano and are described below.

Polymerase chain reaction (PCR). Site directed mutagenesis was performed using PCR to also amplify the mutated DNA containing the double point mutation. The QuikChange Lightning Site-Directed Mutagenesis Kit from Stratagene and its protocol were adapted. All procedures were performed under sterile conditions. Forward and reverse primers containing the mutation of interest were obtained with the kit. A 10-fold dilution was done on both primers in sterile water prior to preparation of PCR solution. A 50 µL solution containing forward and reverse primer, sterile water, plasmid, QuikChange XL dNTP Mix, 10x QuikChange Lightning reaction buffer, QuikChange solution, and QuikChange Lightening Enzyme was prepared in a PCR Eppendorf Tube. The tube was then placed in the PCR machine for approximately four hours under the following PCR conditions:

Step	Number of Cycles	Temperature (°C)	Time (seconds)
1	1	95	120
2	30	95 (denaturation)	20
		55 (annealing)	30
		65 (elongation)	750
3	1	65	300

A final enzyme was added following the completion of PCR involved in the mutagenesis to digest the initial parent DNA strands.

Plasmid Preparation. The QIAprep Spin Procedure from the QIAprep Miniprep Handbook was used to prepare plasmids with the DNA of interest amplified in the PCR procedure. Heat Shock was used to transform the plasmids into *E. coli* BL21 DE3 cells for overexpression. Colonies that grew on plates were used to prepare glycerol stocks.

Cell expression. On day one of the cell expression process, two 2-liter flasks of LB were prepared by adding 25.0 g of LB to 1.00 L of deionized water into each flask. The autoclave was used to sterilize the two flasks containing LB as well as Eppendorf tubes and pipette tips. Following the completion of sterilization, two cell cultures were prepared to obtain fresh, dense stock of cells. The first cell culture consisted of *E. coli* BL21 cells with BM3 wild-type and the second cell culture was of the BM3 A82F F87A mutant. Three falcon round bottom tubes were prepared by mixing 5 mL of sterile LB and 5 uL of ampicillin (Ampicillin Sodium Salt, Fisher BioReagents) at a concentration of 100 mg/mL. One falcon tube was designated as the negative control of cell growth and contained no addition of cells, while 5 µL of the glycerol stocks were added to each of the other two falcon tubes. The cell culture was then placed in the C24KC Refrigerated Incubator Shaker to mix at 225 rpm at 37°C overnight to stimulate cell growth. After 12 hours, 3 mL of the overnight cultures, 1 mL of the 100 mg/mL ampicillin, 20 mL 1 mg/mL of hemin in glycerol stock were added to 1 Liter of LB and allowed to grow at 37°C and 225 rpm until the optical density at 600 nm was between 0.6-0.8. To induce protein expression, 238 mg of isopropyl-β -D-1-thiogalactopyranoside (IPTG - a protein inducing agent) were added into each culture and were then placed in the same shaker overnight. On the third day, the grown cells were centrifuged with the Avanti J-26S XP Centrifuge at 5000 rpm for 30 minutes at 4°C. This allowed for separation

of the cells from the LB broth. The cells were then suspended with 50 mM Tris with 20% glycerol solution and placed in a -80°C freezer. The next day, the frozen cells were thawed on ice and then 0.1 M of phenylmethanesulfonyl fluoride (PMSF), a serine protease inhibitor, was added to the defrosted cells. The cells were then lysed by three passes through a French Press at 15,000 – 18,000 psi. The lysed cells were then centrifuged for 30 minutes at 17,000 rpm. This allowed for separation of the lysed cells from the protein of interest. Glycerol was added to the centrifuged protein and stored in the -80°C freezer for purification.

Purification. A DEAE-650m column or anion exchange chromatography was first performed to purify the immobilized His_6-tagged WT and mutant expressed protein. First, the column was washed with 30% ethanol solution for ten minutes and then with deionized water for ten minutes to ensure the column was clean and ready for use. Then, 50 mM Tris-HCl buffer with pH of 7.4 was run on the column. The thawed protein was then loaded onto the column and then run with 50 mM Tris-HCl with pH of 7.4 again for ten minutes. The column was then washed with 50 mM Tris/100 mM NaCl pH of 7.4 for ten minutes. Then, to elute the protein of interest off the column, a higher salt concentration buffer of 50 mM Tris / 250 mM NaCl pH of 7.4 was added to begin elution and collection. The most concentrated fractions dialyzed overnight in a 50 mM Na_3PO_4 pH = 7.4 to remove excess salt. The dialyzed protein was then run on a second column; a Nickel column (IMAC). The column was again cleaned with 30% ethanol and water and then finally with equilibration phosphate buffer (50 mM Na_3PO_4 / 100 mM NaCl at pH of 7.4). Then protein was loaded and then equilibrated with the same phosphate buffer for about ten minutes. Then, it was washed with a low imidazole concentration buffer (50 mM phosphate / 100 mM NaCl / 25 mM imidazole at pH 7.4). Then, to finally elute the protein, a higher concentration imidazole was used (with 200 mM imidazole). The most concentrated fractions were collected and centrifuged with

phosphate buffer several times to wash off the imidazole and further concentrate the protein. The final product was aliquoted and stored for later use.

Quantification and determination of purity and protein concentration. UV-vis spectrometry was used to determine the efficiency of the expression and protein purification. The scan mode of the spectroscopy was used to determine the wavelength at which the purified protein absorbs. In a 1 cm cuvette, 2000 uL of 50 mM K_3PO_4 buffer pH=7.4 was mixed with 100 µLs of protein and the wavelengths from 700 nm to 250 nm were scanned. If the imidazole was completely washed off the protein, P450 should absorb at λ_{max} = 418 nm. If the maximum absorbance was observed at 424 nm, the protein was centrifuged again with 50 mM K_3PO_4 buffer pH = 7.4. Beer's law was then used to calculate the concentration of the protein collected using the absorbance obtained at the maximum wavelength and the molar absorptivity of the enzyme which is 94,500 M^{-1} cm^{-1} and 1 cm path length. SDS page gel was also used to determine the purity of the protein.

Solution Preparation. Various solutions were prepared for the experiments. Arachidonic acid, eicosanoic acid, and palmitic acid solutions were all prepared in 50 mM K_2CO_3 at a concentration of 780 µM and placed in a 50°C water bath to improve solubility. In addition, the potassium phosphate buffer was prepared at 50 mM phosphate concentration pH = 7.4. The NADPH stock was also prepared in deionized water carefully due to NADPH's sensitivity to obtain a concentration of 13.4 µM (or 10 mg/mL).

Kinetic studies. To determine the catalytic activities of both the WT and mutated enzyme, kinetic studies were conducted using the UV-vis Spectrophotometer with a water bath. The assays were performed at 15, 25, and 35°C. In the 1 cm cuvette, 2000 µL of buffer, 15 µL of NADPH, 10 µL of protein, and substrate with varying volumes (1-300 µL) was used to determine the activity of the enzyme with each substrate. The buffer used as the solvent and to obtain the blank

measurement for all the experiments was a 50 mM potassium phosphate buffer (K_3PO_4). The concentration of the protein in the cuvette varied in concentration but was mostly contained at approximately < 1 µM. A stock of all three substrates at a concentration of 780 µM was prepared to use for the kinetic assays, and its concentration in solution varied from 0.385 – 116 µM. The absorption wavelength was set at 340 nm and the depletion of NADPH over time was monitored. Beer's Law and Michaelis Menten kinetics were used to manipulate the data and IGOR Pro software was used to best fit the various plots and to calculate the kcat and Km values for each of the kinetic assays to allow for calculation of the catalytic efficiency for each substrate. The substrates were kept in a water bath at 48°C to control their solubility.

Percent high spin studies. To determine the percentage of high spin of arachidonic, eicosanoic, and palmitic acid with the wild-type and mutant P450, the scan mode of the UV-vis Spectrophotometer was used. These studies were carried out with 50 mM K_3PO_4 buffer at pH 7.4. For each study, the protein concentration used was about 2-3 µM in the solution and the solution was then saturated with substrate at a concentration that is 100x the protein concentration in the solution or 2x the Km obtained from the kinetic assays. A scan of the solution was taken from 700 to 250 nm and the absorbance values at 390 nm and 418 nm were recorded for all studies with temperatures ranging from 15 to 35 °C. The Fisher *et al.* paper was used to help with the final calculation of the percent high spin at each temperature using the absorbance at 390 and 418 nm and was then finally plotted for further analysis (*10*).

Arrhenius plots. To determine the activation energy of the various reactions, Arrhenius plots corresponding to each study were produced. The natural logarithm of the kcat of each kinetic assay was plotted on the y-axis and the reciprocal of the temperature of each kinetic assay in Kelvin was plotted on the x-axis. The slope (m) of each plot is equal to $m = \frac{-Ea}{R}$ where R is the universal

gas constant (R = 8.3145 $\frac{J}{K\ mol}$) and Ea is the activation energy. The best fit line and equation of each Arrhenius plot made it possible to calculate the activation energy associated with the P450 chemical reactions.

Results and Discussion

We hypothesized that the double mutant would increase the percentage of the substrate in the proximal conformation by directing the substrate away from the hydrophobic pocket. This was tested by monitoring the percent high spin by UV-vis spectroscopy. Kinetic assays using UV-Vis spectrometry were conducted for wildtype cytochrome P450 and mutant A82F F87A. Six assays in total were carried out for each substrate, three with the WT and the other three with the mutant. Also, each assay was conducted at a different temperature to study the temperature dependency of the enzyme. The temperatures used were 15°C, 25°C, and 35°C. The curve fittings from the IGOR software also allowed for the calculation of the kcat and Km for each assay. The data obtained from the curve fittings of the kcat plots as well as percent high spin tests carried out made it possible to conduct quantitative and qualitative analysis of the catalytic activity of WT and mutant P450.

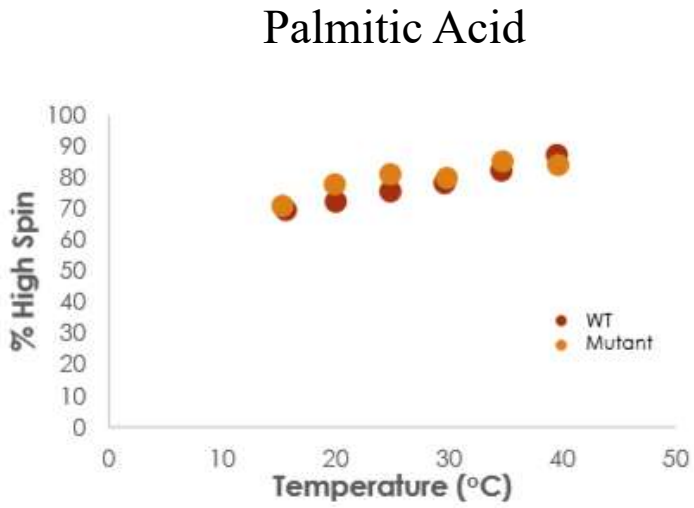


Figure 3. The percent high spin test of the WT and mutant P450 with palmitic acid (PA) as the substrate. The percentage of high spin increases with increasing temperature for both proteins and relatively a similar trend is seen with both fittings.

Palmitic Acid

The percentage of high spin state correlating to the active proximal conformation increased with increasing temperatures for studies with palmitic acid as the substrate. Figure 3 shows that there is a slight increase in the percent of high spin state for the mutant than for the WT at all temperatures, except 35°C. Previous studies by Geronimo *et al.* showed a percent high spin of palmitic acid with WT P450 at 25°C to be 94 (±10) % which agrees with the presented results (*1*).

Table 1. kcat, Km, and catalytic efficiency of WT and A82F F87A CYP450 with palmitic acid for temperatures ranging from 15-35 °C.

	Temperature	K_m (μM)	k_{cat} (s^{-1})	**k_{cat}/K_m (1/s μM)**
WT	15 °C	7 ± 2	3.5 ± 0.3	**0.49 ± 0.15**
WT	25 °C	19 ± 4	8.0 ± 0.9	**0.42 ± 0.10**
WT	35 °C	16 ± 3	8.3 ± 0.6	**0.53 ± 0.10**
AF Mutant	15 °C	23 ± 5	4.3 ± 0.3	**0.19 ± 0.04**
AF Mutant	25 °C	22 ± 4	5.8 ± 0.4	**0.27 ± 0.05**
AF Mutant	35 °C	29 ± 8	10.9 ± 1.6	**0.37 ± 0.12**

Km is the substrate concentration at which half of the active sites of the enzyme molecules in solution are occupied. Larger Km values indicate lower affinity to substrate and thus greater concentration of the substrate is needed to achieve the reaction rate when the enzyme is fully saturated with enzyme (at V_{max}). The turnover number, kcat, is also important for determining the catalytic efficiency of the enzyme and it is equal to $\frac{V_{max}}{[Enzyme]_{total}}$. The catalytic efficiency is calculated by dividing kcat by Km for each kinetic assay. According to table 1, there is an increase in the catalytic efficiency of the enzyme with increasing temperature. In addition, a higher catalytic activity is observed for the WT kinetic assays than the mutant kinetics. This finding appears to be due to a lower affinity (larger Km) of the substrate to the double mutant, thus decreasing the overall catalytic activity of the mutant. Palmitic acid was a good substrate of P450 compared to the other fatty acids studied due to the overall higher catalytic efficiency observed in all its kinetic assays with the wildtype and mutated P450. Its solubility issue was resolved with the use of the water bath throughout the assays. Greater binding of PA with wildtype compared

to the mutant is seen in Table 1. In addition, the results obtained from the kinetic studies do not completely agree with the percent high spin results. According to the percent high spin study, wild-type was only more efficient than the mutant P450 with PA at 35°C, however, according to the catalytic efficiencies calculated in Table 1, the wild-type enzyme is more efficient with PA as substrate for all temperatures. P450 BM3 has previously shown to favor the catalysis of saturated fatty acids that consist of 14 to 26 carbon chain lengths for the NADPH dependent hydroxylation (*11*). The kinetic assays with palmitic acid compared to those with eicosanoic acid as the substrate showed higher catalytic efficiency and a greater turnover number for all the P450-catalyzed reactions and thus although P450 BM3 favors a wide range of saturated fatty acids, the results showed a greater preference towards the shorter carbon chain fatty acid.

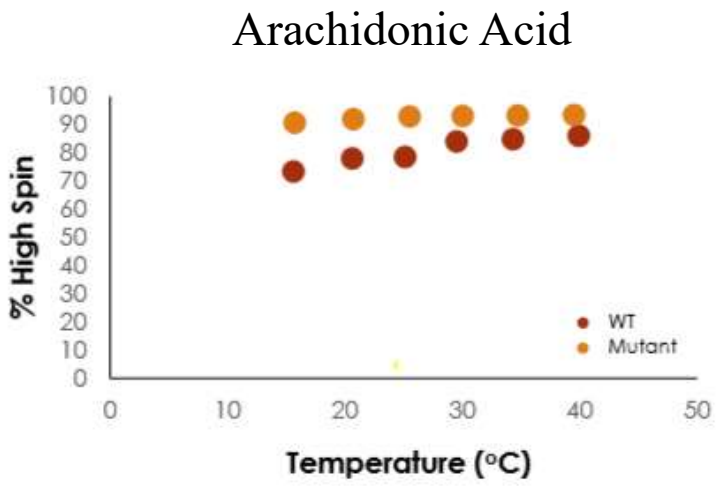


Figure 4. The percent high spin as a function of temperature for the substrate, arachidonic acid (AA), with both the WT P450 and mutant P450. Percent high spin increase with increasing

Arachidonic Acid

There is a greater increase in the percentage of high spin state at all temperatures with arachidonic acid and A82F F87A mutant than with the WT P450, as shown in Figure 4. In addition, the percentage high spin increases for both the double mutant and WT P450 as a function of temperature. The double mutant has a higher percentage of the substrate in the proximal conformation with arachidonic acid as the substrate than palmitic acid according to the percent high spin plots.

Table 2. kcat, Km, and catalytic efficiency of WT and A82F F87A CYP450 with arachidonic acid (AA) for temperatures ranging from 15-35 °C.

	Temperature	K_m (μM)	k_{cat} (s^{-1})	**k_{cat}/K_m (1/s μM)**
WT	15 °C	11 ± 2	2.1 ± 0.2	**0.20 ± 0.04**
WT	25 °C	26 ± 4	7.0 ± 0.5	**0.27 ± 0.04**
WT	35 °C	10 ± 3	6.0 ± 0.5	**0.58 ± 0.18**
AF Mutant	15 °C	13 ± 3	1.7 ± 0.1	**0.13 ± 0.03**
AF Mutant	25 °C	23 ± 4	3.4 ± 0.2	**0.15± 0.03**
AF Mutant	35 °C	29 ± 10	5.3 ± 0.9	**0.19 ± 0.07**

The catalytic efficiency is highest for the kinetic assay of WT-AA at 35°C, as shown in Table 2. Again, an increase in the catalytic efficiency is observed with increasing temperatures. The WT P450 catalytic efficiencies are approximately 2x those of the mutant with AA, which is similar to what was seen for palmitic acid. There is no correlation between the kinetic assay results and the percent high spin studies illustrated in Figure 4. Arachidonic acid showed the highest efficiency and catalytic activity with the wild-type enzyme than with the double mutant. Previous

studies indicate that cytochrome P450 BM3 displays one of the highest catalytic activities with arachidonic acid as the substrate (*11*).

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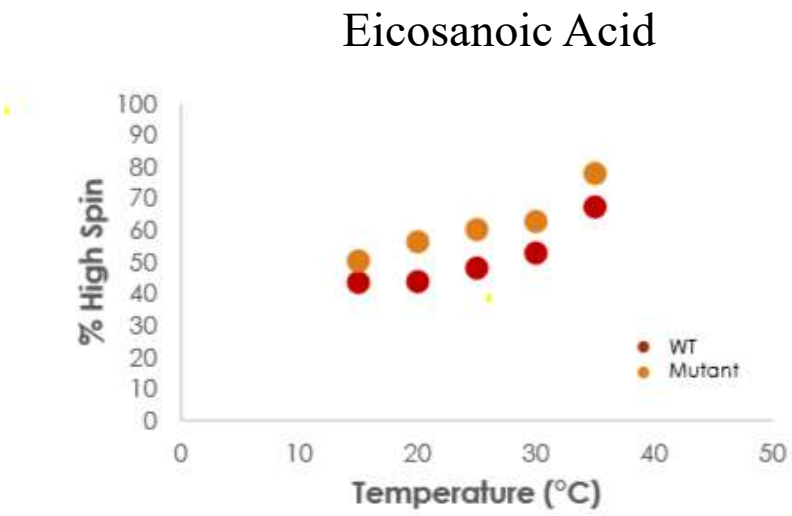

Figure 5. The percent high spin as a function of temperature for the substrate, eicosanoic acid (EA), is higher for the mutant than for the WT P450.

Table 3. kcat, Km, and catalytic efficiency of WT and A82F F87A CYP450 with eicosanoic acid (EA) for temperatures ranging from 15-35 °C.

	Temperature	K_m (μM)	k_{cat} (s^{-1})	**k_{cat}/K_m (1/s μM)**
WT	15 °C	20 ± 7	0.8 ± 0.2	**0.04 ± 0.02**
WT	25 °C	31 ± 10	2.6 ± 0.3	**0.09 ± 0.03**
WT	35 °C	25 ± 5	3.5 ± 0.2	**0.14 ± 0.03**
AF Mutant	15 °C	29 ± 15	1.5 ± 0.4	**0.05 ± 0.03**
AF Mutant	25 °C	47 ± 12	3.6 ± 0.4	**0.08 ± 0.02**
AF Mutant	35 °C	63 ± 13	8.3 ± 1.0	**0.13 ± 0.03**

Eicosanoic Acid

According to Table 3, there was not a clear difference in catalytic efficiencies between the wild-type and mutant P450 with eicosanoic acid as the substrate. The catalytic activity does, however, again increase with increasing temperatures. The overall enzymatic reaction rate for all the kinetic assays with eicosanoic acid as the substrate are lower than those of arachidonic and palmitic acid. Eicosanoic acid being a saturated 20-carbon chain fatty acid is not a favorable substrate of P450 BM3. Furthermore, Figure 5 indicates that the percentage of high spin of the double mutant is larger than that of the WT for all temperatures, however, the difference is not significant and does not agree with the kinetic assays and thus a firm conclusion cannot be confirmed.

Table 4. The activation energy (E_A) of the WT and mutant P450 catalyzed chemical reactions derived from the Arrhenius plot of each substrate.

	EA - WT	**EA - AF**	**AA - WT**	**AA - AF**	**PA - WT**	**PA - AF**
Activation Energy (E_A), kJ/mol	54.8	63.1	39.3	42.0	32.2	34.2

Since kinetic data was obtained as a function of temperature, it was possible to determine the activation energy of each substrate. The proposed activation energy associated with P450 chemical reactions suggest that the activation energy may range from 30–70 kJ/mol (*12*). According to Table 4, the activation energies of the catalyzed reactions ranged from 34-63 kJ/mol, and thus they agree with previous studies. There is consistency in the range of activation energy calculated for each two catalytic reactions with each substrate.

When phenylalanine at position 87 near the heme or active site was mutated to valine, the amino acid replacement converted P450 BM3 into a stereoselective-arachidonic acid epoxygenase and did not inactivate the enzyme (*11*). The mutant displayed similar catalytic behavior as the wildtype P450 BM3. When F87 was replaced with an alanine in this study, however, the enzyme remained active as indicated by the catalytic activities in Table 2, however, the activity showed a decrease from when the wild-type studies were conducted, which shows that the decrease in activity may be due to A82F. Mutations of A82F have shown a decrease in Km for palmitate, and comparable kcat which leads to an increase in catalytic activity (*2*). Mutations of A82F have also shown an increase in high spin for palmitate compared to the wildtype (*2*). Furthermore, a double mutation of A82V and F87A has shown an increase in catalytic activity that was not seen with double mutant A82F F87A from this study, indicating that possibly the mutation of residue 82 to a phenylalanine causes a decrease in activity.

Conclusion

The percentage of high spin state at temperatures ranging from 15-35°C shown in Figures 3-5 indicate that BM-3's percentage of high spin state increases with increasing temperature, and thus it is highest for all substrates at 35°C. Percentage of high spin state corresponds to the proximal ligand conformation of P450 which is the necessary state for catalysis to occur, thus, I expected to see more product formation and greater hydroxylation of the substrate than at lower temperatures. This trend was observed for reactions catalyzed by both the wildtype and mutant P450 with all the substrates. Correspondingly, kinetic assays performed under defined conditions also supported such spin state temperature dependent findings and, indeed, greater product

formation with increasing temperatures was observed. This was supported with an increase in catalytic activity (kcat/Km) as seen in Tables 1-3. Greater catalytic efficiency could also indicate an overall greater percentage of the proximal ligand conformation that is necessary for catalysis.

Additionally, although a temperature dependence of the catalytic efficiency of P450 was observed, my proposed hypothesis was not supported. I proposed that the double point mutation of P450 on amino acid residues 82 (alanine) and 87 (phenylalanine) would show an increase in the percentage of high spin state and in the catalytic activity of P450 BM-3. The mutant did show an increase in the percentage of high spin state, however, according to Tables 1-3, higher catalytic activity of the enzyme was observed with the wildtype P450 rather than the mutant A82F F87A P450. Although the phenylalanine was moved a few amino acids away from the active site, its effect on the attraction of the hydrophobic fatty acids might not have changed. It has been suggested that although the removal of phenylalanine 87 could solve the hydrophobic pocket issue, the increased space created by removing such a large residue from near the active site could result in reduced coupling of NADPH as well as increased mobility of the bound substrate in the active site, leading to decreased regiospecificity and as a result, decreased catalytic activity (*2*). Also, previous studies that performed a single amino acid mutation on only F87 by changing it to valine suggested 100x greater hydroxylation of β-ionone, a chemical compound found in many essential oils (*13*). This finding suggests that removing the phenylalanine completely from near the active site allows for bulkier substrates which can improve the overall efficiency of P450 by increasing hydroxylation for non-natural substrates. My results, however, suggest that the phenylalanine residue near P450's heme greatly contributes to its activity and its conformational dynamics upon coming in to contact with substrate.

Although A82F F87A P450 BM-3 did not show an increase in the hydroxylation of fatty acids, a fatty acid dependence was observed. Both palmitic acid and eicosanoic acid are saturated fatty acids, but they differ in carbon-chain length. Palmitic acid has a smaller chain consisting of a 16-carbon backbone while eicosanoic acid is larger, with a 20-carbon chain backbone. The catalytic activity of both wildtype and mutant P450 is significantly larger with palmitic acid as substrate than when eicosanoic acid is the substrate. This suggests a chain length dependence on the rate of hydroxylation of P450. Also, when comparing the catalytic activities found with eicosanoic acid as the substrate versus when arachidonic acid, a 20 carbon long fatty acid with four double bonds, as the substrate, another significant change was observed. Greater P450 efficiency with arachidonic acid was seen than with eicosanoic acid. This may indicate that the unsaturation in arachidonic acid caused the increased efficiency in P450, thus, suggesting that P450 is also dependent on the saturation of fatty acids. Further studies must be carried out, however, to confirm the findings of chain length and saturation level dependence of fatty acid hydroxylation by P450s.

Eicosanoic acid displayed the largest activation energy, as seen in Table 4. Thus, further illustrating that eicosanoic acid was the least favorable substrate for P450 tested. According to Figure 5, eicosanoic acid also had an overall lower percentage of high spin state for both wildtype and mutant P450 compared to arachidonic and palmitic acids. Palmitic acid showed the greatest catalytic activity of P450. However, further research must be done to confirm if this behavior is solely based on chain length dependence.

Aside from conducting further studies with other fatty acids, future studies also include testing the efficiency of P450 with different classes of substrates. The natural substrate for P450 is unknown, but P450s can catalyze reactions with a wide variety of substrates. I would want to

study how the catalytic efficiency of P450 varies from different types of substrates. For example, the double mutation F82V and F87A coupled with other mutations opens the binding to allow for a wide variety of substrates (*14,15*). Furthermore, I want to study the hydroxylation products of fatty acids using Mass Spectrometry and HPLC. Previous studies suggest that wildtype P450 hydroxylates fatty acids at the ω-1, ω-2, or ω-3 positions on the fatty acid. However, a mass spectrum of A82F F87A with lauric acid (C12:0) as the substrate was obtained and showed possible hydroxylation at the ω position. However, further testing must be carried out, specifically with the three fatty acids in my study, to further explore the products of fatty acid hydroxylation by the double mutant P450 to better understand the catalytic activity and ligand conformation seen in this study.

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