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## Cytochrome P450 3A4: The Impossible Protein



### Introduction

It has been a difficult experience working the liver protein known as P450 3A4. This particular protein is found in the liver membranes, and because of its nature to adhere to membranes, the protein is not an easy one to purify. While most proteins only need a few specific purification steps, I have had the opportunity to work with nearly every purification technique that I had learned about in the Biochemistry class I took during my experience with P450. I specifically remember the professor saying he did not want to get into the topic of membrane proteins because they were “a big mess.” Now I completely understand what he meant.

The technology in the realm of optics and physical chemistry has the potential to unlock important scientific facts about proteins such as P450s. A drug that goes in to the body is not always the same one that causes the useful (or harmful) effects. For instance, codeine is turned into morphine and benzene itself is not a carcinogen but in the oxidized form created by the P450 enzyme it is harmful to human DNA. Doctors and pharmacists are still unable to predict how certain drugs will react when administered together because of the uncertainty in the way P450 might react with both of these drugs at once. It is speculated that the specific P450, 3A4, can bind two drugs at the same time, and in

this situation the drugs will not be oxidized in the same way that they would be if there were only one bound to the protein. This makes it difficult to administer more than one drug at once because of the possible effects that occur due to 3A4. In addition to having this binding property, 3A4 is the most abundant cytochrome P450 in the liver. It is very important to drug metabolism in humans, thus making it an important protein to do research on.

## II. The Protein

The protein P450 3A4 is one of the most abundant P450s in the liver. It is unique in that it has a particularly large binding cavity which could possibly hold more than one drug at once. Although this has never been proven, the closely related bacterial P450 eryF has been shown bound to two ligands at once. It is what lies within the binding cavity that allows the protein's binding habits to be studied more in depth.

The first key factor in being able to determine the binding of the protein is that it contains a heme group in the binding cavity. A heme is a highly conjugated chemical group with an iron atom bound in the center. The conjugated structure of the heme is what allows it to absorb light. The more conjugation that occurs in a chemical compound, the closer the HOMO (highest energy bonding pi-orbital) and LUMO (lowest energy anti-bonding pi-orbital) become. Since these two orbitals become closer with more conjugation, a highly conjugated complex, such as a heme, can absorb light easier, and will usually absorb in the UV/VIS range. The protein 3A4 absorbs light at around 416 nm, although this can change between the oxidized and reduced state. When a ligand is bound to the protein, the wavelength at which the protein absorbs light becomes shorter. With this effect, a difference spectra can be obtained. A difference spectra is the difference that arises between the unbound absorbance peak of the and the bound absorbance peak as the concentration of the ligand increases. A plot of the differences can be analyzed to determine whether there is cooperative binding taking place or not.

The second important factor that helps to determine how the protein binds is that there are two tryptophan amino acid residues close to the binding cavity and positioned on either side. Tryptophan is a planar amino acid that will fluoresce a small amount when it is excited by a wavelength of about 280nm. The fluorescence always occurs at a longer wavelength than that which the protein was excited at because some vibrational energy is lost to the surroundings. In the case of tryptophan, the fluorescence occurs at around 350 nm. When a ligand binds near the tryptophan, the fluorescence is quenched because the energy from the excited tryptophan is passed to the ligand instead of released. Using this information a binding curve somewhat like the one obtained using difference spectra can be obtained by inverting the fluorescence intensity vs. the ligand concentration plot at about 350 nm. This plot can also determine if cooperative binding takes place after the plot is analyzed. If a tryptophan were to be removed and the binding curve were to be redone, new information regarding where the ligand binds, and if the ligand would still be able to bind, could be obtained.

### **III. The Problems with Purification**

In an ideal world the protein would be pure enough to do accurate difference spectra and fluorescence testing on, and the lasers could be used to determine the actual rate of reaction taking place using fluorescence. However, the protein has been difficult to purify, and has not yet reached a pure enough state in which it could be tested with lasers.

The first step in obtaining the protein is to insert the DNA for the protein into a bacterial plasmid. Certain modifications to the DNA are usually made before insertion. Two that have worked well with P450 3A4 are adding ampicillin resistance and adding histidine amino acids onto an end of the protein. The ampicillin allows for the bacteria to grow in an environment treated with ampicillin where other unwanted bacteria cannot contaminate the culture and grow. The histidine tag will allow for the protein to stick to a nickel column in a purification process that will be described later on. One modification to the DNA that did not work was removing hydrophobic amino acids from the outside of the protein in an attempt to make the protein more soluble in water and to keep it from sticking from the bacterial membranes so much. This resulted in the protein not being expressed in the bacteria well. Another attempt to modify the DNA was to change the two tryptophan amino acids in the binding cavity to phenylalanine amino acids, which are also planar in nature, but only have one ring instead of the two that tryptophan has. This resulted in either very little protein being expressed, no protein being expressed, or a different form of protein that is not like P450 at all being expressed. Either way, we have not yet been able to get a decent amount of mutant protein in this way.

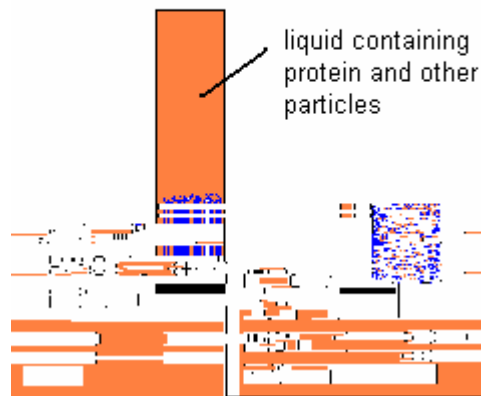
Once the DNA is inserted in the bacteria, the bacteria is turned into stock which can be used to grow many batches of protein containing bacteria. The bacteria is grown for about three days before the protein can be obtained from it. Once this has been done, the purification process begins. This process has been very complicated and the protocol has been changed several times in order to obtain a higher yield of protein, and a purer sample of protein.

The first stage of purification is to spin the cells down, and then resuspend the dead cells. The next step has been used in our lab throughout all of the purifications, but is not used in all labs. This step is the nitrogen freezing step. Some biochemist believe the quickly freezing the cells in liquid nitrogen will disrupt spheroblasts in the cells and allow them to lyse easier. Other labs go directly to the sonication step, which is the next step that is done in our lab.

Sonication causes the cells to burst using a high pitched noise. We have tried this for very long lengths of time and shorter lengths of time. In the end we found that it works best to sonicate for a shorter amount of time. During this step it is important to add

protease inhibitor because it is at this stage that the protein is exposed to all of the harmful elements of the cell such as proteases which will destroy proteins.

The next step is to add a detergent and stir the protein. The detergent helps to break away the protein from the cell wall. After the protein has stirred with the detergent for a few hours, the excess membranes can be spun down. The next step of the purification involves the nickel resin column referred to earlier. The column must first be equilibrated with the buffer being used with the protein. After the column resin is equilibrated it is added to the protein solution and the resin and protein are left to mix on a roller overnight. If this step works correctly, the protein will stick to the resin and the excess proteins and membrane matter will flow through the column and not stick to the resin at all. The protein can be removed from the resin by pouring something through the column with a higher affinity for the nickel resin than the protein such as imidazole. One is left with mostly the protein that is wanted, some protein that might have histidine on the folds, and the imidazole. A diagram of this process is shown below in figure 1.



**Figure 1**

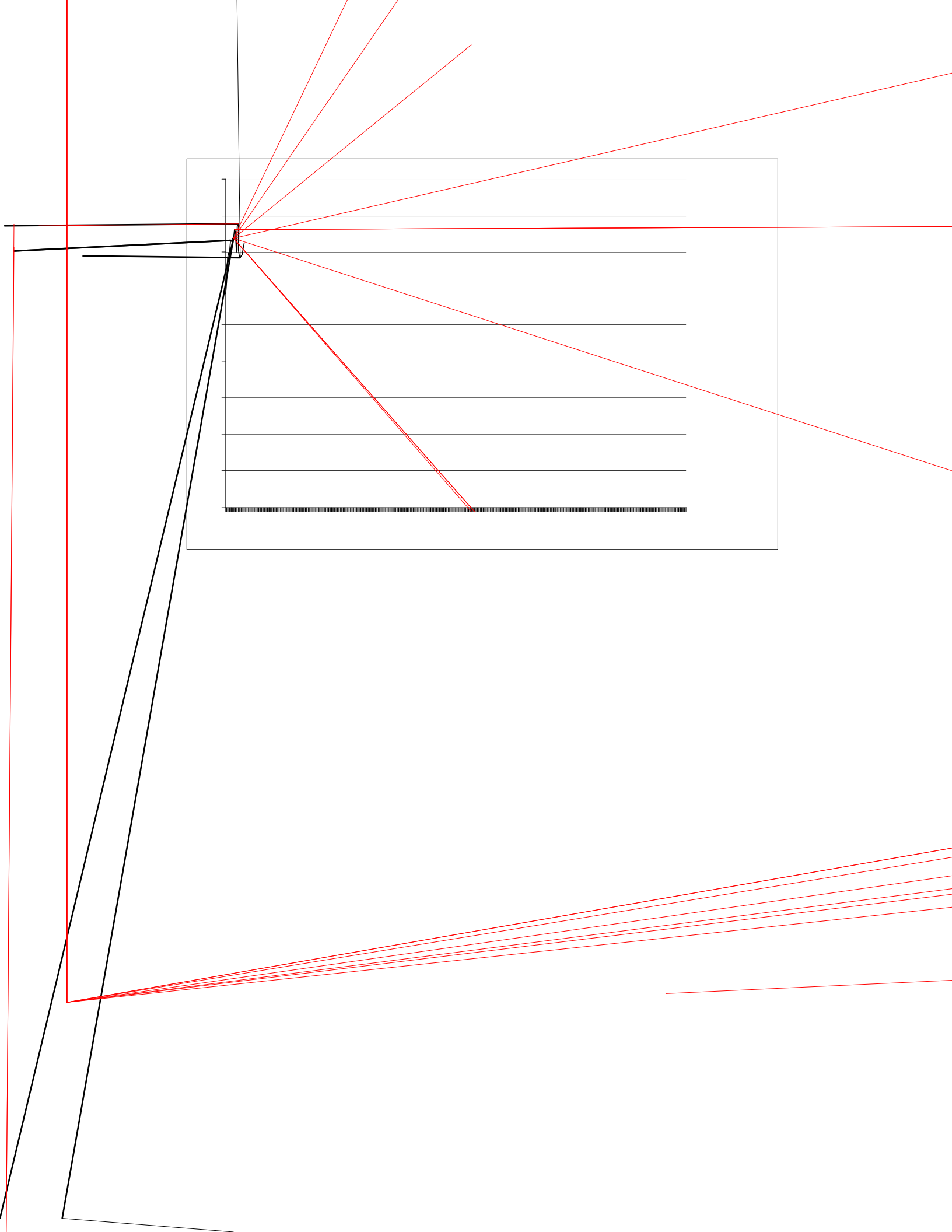
The next step removes the small amounts of remaining protein along with the imidazole. This uses another technique called cation exchange chromatography. It uses resin like in the nickel affinity chromatography, however this time it is the charge of the protein which allows it to stick to the column. The effectiveness of this process to purify the protein has been shown through the absorbance spectrum and gel electrophoreses performed in our lab. The purity of a protein as compared to samples that have not gone through this process is very large. However, with this wonderful purity comes consequence, the process at best has only had a 50% recovery rate at best when it is performed in our lab. This process has not even worked every time, and the cause for the times that it has not worked at all is still not completely clear.

Throughout the purification, the mixture of buffers and chemicals that the protein is kept in is extremely important. Of all the things we have changed in our procedure, the solutions the proteins are kept in are what have changed the most. During my time working with P450 3A4 I have used the same protease inhibitor and the same detergent. However, the buffers, salts, and protein preservatives used have all changed.

The first buffer that was used with the protein was potassium phosphate. This had been used in the procedure for over a year until it was recently suggested that this particular buffer with small ions may be harmful to the protein. There are two buffers that are currently being experimented with, these are MOPS and HEPES. It is still unclear as to which of these works but, both have larger ions that would not be as detrimental to the protein. The salts used have been potassium chloride and sodium chloride, both of these seem to work the same. There have been three protein preservatives, DTT, EDTA, and BME. Of these, BME seems to work the best. EDTA should not be used before the nickel column because it reacts with the column, so it has proven easier not to use it.

When the purity of the protein is referred to, the methods of determining this are gel electrophoresis, and the absorbance spectrum. With gel electrophoresis, the proteins are pulled down a gel according to size. For a very pure protein there will only be one distinct line of protein. When the protein is not as pure there will be other bands in the gel. Before cation exchange was performed on some of our samples the gel electrophoresis showed other bands besides the P450 band. After the cation exchange was run, the P450 was the only extensive band in the gel. The two tryptophan mutants showed a very light band where the P450 should be and several darker bands in other places indicating that the mutants are not yet at a pure enough stage to work with.

The absorbance indicates the purity of the protein by showing if there are absorbance peaks in other places besides where the heme would usually absorb or if there is any background absorbance. A peak at 280 nm indicates either protein absorbance or excess imidazole. Theoretically the ratio of the peak at 416nm to the peak at 280nm should be a 1.6:1 ratio. The absorbance spectra of P450 before the cation exchange and after the cation exchange are shown in figures 2 and 3 respectively.



From the spectra it is apparent that there was a notable decrease in the peak at 280nm and thus an improvement in the purity of the protein. The yield of this particular protein was about 50% of the original amount put in with the cation exchange beads. Once this step of purification is refined, it will be very beneficial.

#### IV. Results and Analysis

Some of the protein from the purification process was used to find difference spectra and a binding curve through fluorescence. This difference spectra was the first step in determining the binding behavior of the protein. The goal of this step was to get our results to match the published difference spectra results for P450 3A4 to ensure the protein we are working with is as pure as the protein used in published data.

An important equation involved in analysis of this data is known as the hill equation shown below in equation 1.

$$V(s) = V \max\left(\frac{[S]^n}{(K^n) + ([S]^n)}\right) \quad (1)$$

In the above equation 'n' represents the cooperativity of the binding, K is the dissociation constant, Vmax is the max emission or absorbance, and S is the substrate. The variable 'n' should usually be a whole number such as 1,2,3... and if it is greater than 1 there is cooperative binding and equal to 1 means there is no cooperativity. In the case of P450 3A4, 'n' should be about 2. Realistically 'n' is a little bit less than 2 because of there is always protein that is slightly denatured or does not work properly. Protein could still even be stuck to the membrane causing the results to be imperfect. The results of a difference spectra binding curve are shown in figure 4.

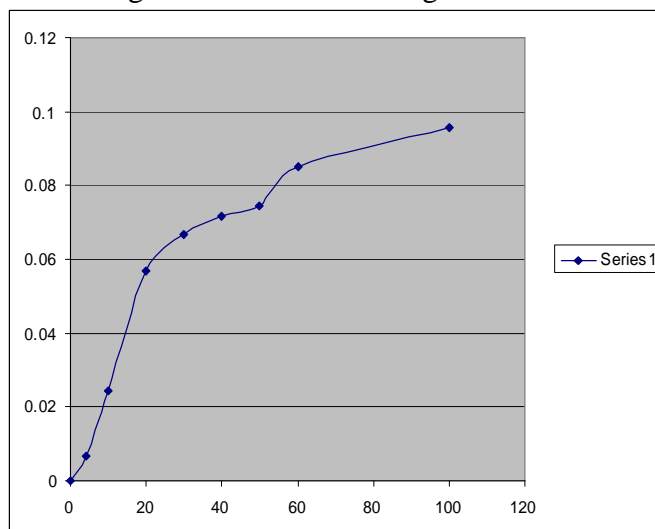


Figure 4

The above data were fitted to the hill equation using origin pro. K was found to be 22.41, n was 1.18, and Vmax was found to be .11049. These data do somewhat drift from what would be expected. This is most likely because the protein was not at a pure enough stage. Also, at higher concentrations of ANF, the protein will form a precipitate, causing the absorbance to increase. Precipitation usually begins to occur when the concentration of ANF is about 60uM. This can clearly be seen from the above plot. However, the difference spectra at higher concentrations are still important because they show what Vmax should be. If the highest point were to be removed, a more realistic value of n, 1.69 is found. Hopefully with a purer sample of P450, better data can be taken.

The fluorescence plots can also be analyzed with the hill equation. Below in figure 5 is a plot of the tryptophan fluorescence intensity versus the concentration of alpha-naphthoflavone ligand. In figure 6 is a plot of this inverted and normalized so that the hill equation can be fit to the curve. The curve must be inverted to fit the data to the hill equation. The tryptophan fluorescence is quenched by the alpha naphthoflavone, so the fluorescence decreases as the concentration of the alpha naphthoflavone increases. Figure 7 shows the emission spectra of samples of protein with different concentrations of alpha-naphthoflavone.

Fluorescence Intensity (cps) versus ANF concentration (uM)

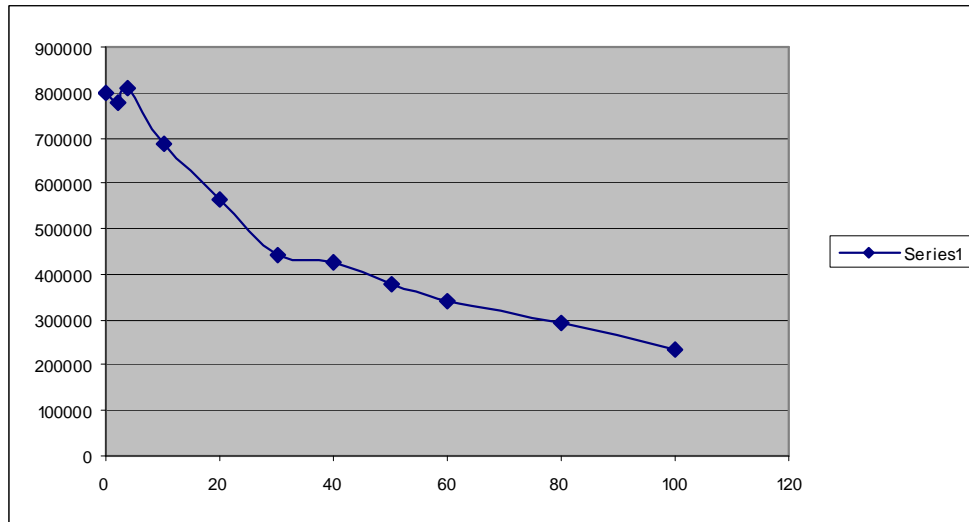
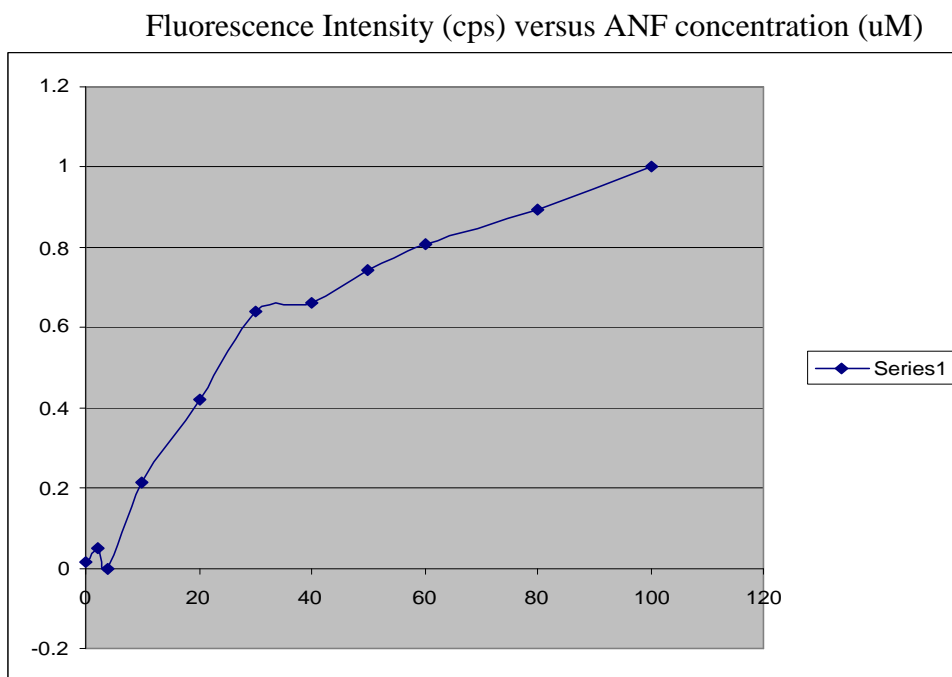


Figure 5



**Figure 6**

When the above data were fitted using origin pro,  $n$  was found to be 1.05. The samples used for these data are the same that were used in the absorbance data. There are several explanations for why the data for tryptophan fluorescence is not the same as the data for absorbance. This is where the mutants will come into play. If the mutant proteins having either of the two tryptophans changed are purified, the way that they react will tell more about how the protein binds to alpha naphthoflavone. Also, the amount of alpha naphthoflavone that is unbound and fluorescing at 340 nm has not yet been taken into account.

In figure 7 it can be seen that as the amount of alpha naphthoflavone increases, the tryptophan fluorescence peak at 340 nm decreases as the fluorescence becomes quenched by the bound ligand.

Emission spectra at different alpha naphthofavone concentrations

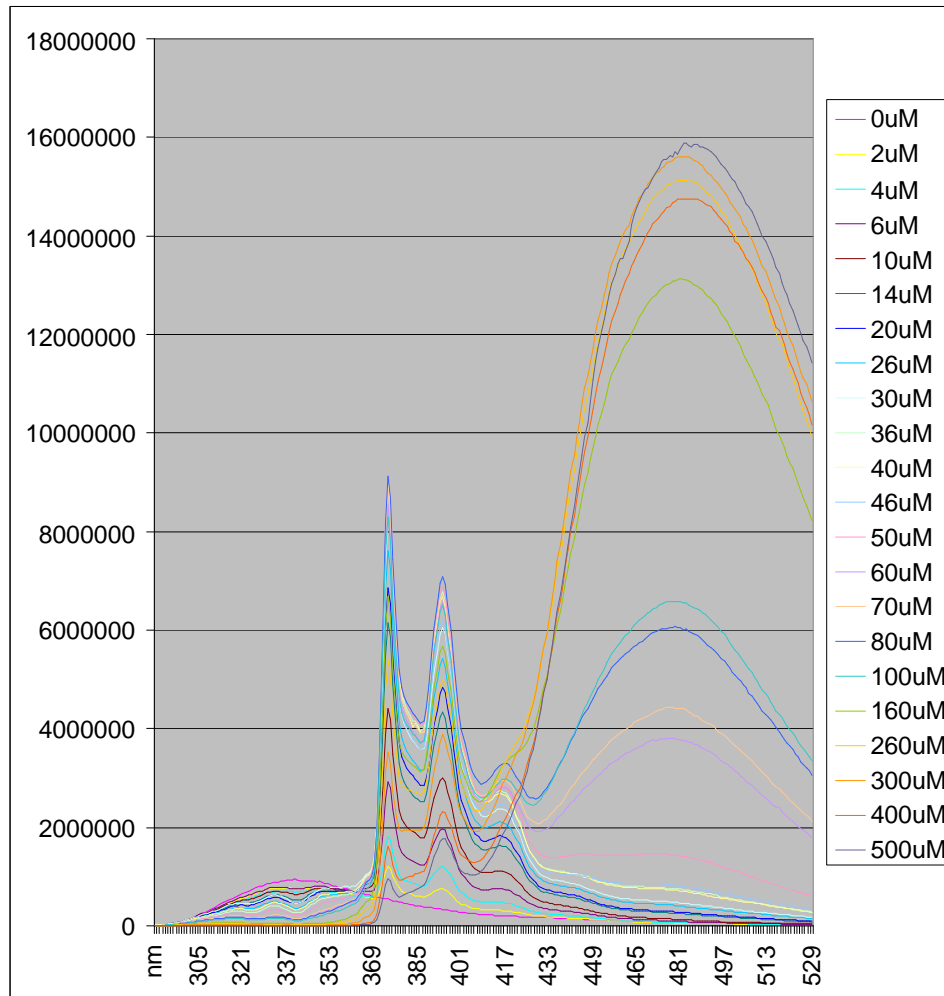


Figure 7

## V. Conclusion

The protein P450 3A4 still has more purification needed before proper testing can be done. The protein is one of the most difficult types of protein to study. It has some parts that ideal for studying fluorescence and absorbance. The heme will change the wavelength that it absorbs at when a ligand is bound to the protein. More about the binding of the protein can be understood by the fluorescence quenching that takes place. If the mutant P450s can be properly expressed and purified, then even more studies on the binding can be done.