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Generating new Type II protein models using cytochrome c

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GENERATING NEW TYPE II PROTEIN MODELS USING CYTOCHROME *C*

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Introduction

One of the most important components of life are large molecules called proteins, used for carrying out an immense variety of biological functions. In addition to their main structure, some proteins have additional, nonprotein components that are necessary for their function. One example of this is the compound heme (Figure 1), which is integrated into the protein structure and used as a binding site. A well---known example of a heme protein is hemoglobin, a protein in the blood that is responsible for transporting oxygen from the lungs into other parts of the body. The heme compound contains an iron atom in the center, and this is where the oxygen actually binds. However, there are many other protein types that contain a heme molecule, including a specific subset that was the focus of this research. Thiolate---ligated heme proteins contain a heme group that is bound to sulfur in the amino acid cysteine. Included in this group are Type II proteins, which undergo a ligand switch. A ligand is a molecule that binds to another molecule. In this protein, the ligand is an amino acid in the protein, and the iron in the heme is the molecule that it binds to. A ligand switch occurs when the original amino acid that is attached to the heme dissociates and is replaced by a second amino acid. For most Type II proteins, the cysteine will be displaced by a neutral ligand, and this controls protein activation and function (Figure 2). The exact mechanism of the ligand switch and how it controls function is not well known in this protein type, and these proteins contain complex structures that are difficult to work with and study.

Purpose

Type II proteins are important because they are implicated in a variety of necessary biological functions, including the binding of gaseous molecules, metabolic processes, and the regulation of circadian rhythms (Table 1). Learning more about the details as to how these biological activities actually work will make it easier to understand how to repair them in various disease states. The purpose of this research was to create a simpler model of Type II proteins in order to study the ligand switch using cytochrome *c* (Figure 3). Cytochrome *c* was chosen because it is small, easy to work with, and relatively easy to mutate. It also already has a built---in mechanism for a ligand switch, making it the ideal candidate for this protein model. Mutating cytochrome *c* into a model Type II protein and learning more about how the ligand switch controls function may generate new insight into how the corresponding ligand switch works in more complex proteins.

Literature Review

The discovery of proteins with a ligand switch involving a sulfur---containing amino acid is relatively new. Omura published a review article in 2005 that describes several mechanisms behind some of these proteins that had just been discovered, including CooA and CBS. CooA is a transcription factor specific to a bacterium, *R. rubrum,* which is able to use this protein to sense the local environment and detect the presence of carbon monoxide. The organism can then manufacture enzymes that allow it to metabolize and survive using carbon monoxide in anaerobic (oxygen deficient) conditions. CBS, or cystathionine beta---synthase, is an enzyme present in many organisms. However, the exact function of

the heme in this important protein is unknown. Studying the mechanisms behind the ligand switch, which greatly affects enzyme activity, may give some insight to how this enzyme is regulated in humans and other eukaryotic organisms.

As more thiolate---ligated proteins are discovered (Table 1), research has shown insight into several patterns related to how these proteins function. Igarashi (*et al*.) explored several key points about these protein types in an article published in 2008. An important aspect of this article that is relevant to the creation of a cytochrome *c* model is the description of the changing oxidation states of the iron in the heme. In these proteins, iron can be in either the +2 or +3 oxidation state, and this can be dependent on the ligands bound to the heme. The proteins can have different properties depending on the state of the iron; therefore, this concept is crucial to understanding the overall mechanism of the ligand switch.

When introducing mutations to a protein, it is important to consider the implications of the changes being made to the structure. A 2012 article by Shimuzu lists several important characteristics of heme sensor proteins that must be taken into consideration when mutating amino acids in this type of protein. Some features of key importance in this research include the binding strength of the heme to the protein, the oxidation state of the iron, and the stabilizing effects of amino acids surrounding the heme.

In addition to finding information about ligand switches in known thiolate---ligated heme proteins, it was necessary to obtain more background information about the protein that will be used for the model, cytochrome *c.* Cherney and Bowler's article, published in 2010, gives an excellent description of the ligand

switch that is already part of this protein's normal functioning. The fact that that cytochrome *c* already had a built---in ligand switch mechanism was one of the key reasons this protein was chosen to attempt to model the more complex Type II proteins.

The primary literature articles provided good background information for several different components of the research to be undertaken, but there were two additional sources of information that pertained more directly to this project. These were the introductory chapters of dissertations from previous graduate students that had worked on similar projects in a lab at the University of Wisconsin---Madison.

The first dissertation was written in 2006 by Samuel Pazicni. The first chapter of the dissertation gave a very useful explanation of what a thiolate---ligated heme protein is. It also explained the differences between Type I and Type II proteins. Essentially, the defining factor of a Type II protein is that the "cysteine ligand is exchange labile," meaning it will readily dissociate and switch with a different ligand. In these proteins there is what is known as a six---coordinate iron, because the iron in the heme forms six bonds, four with the nitrogens in the porphyrin ring, and two with the protein amino acids. A Type I protein will typically have only one ligand to the protein, meaning it is five---coordinate. In addition to explaining the differences between the two types, several examples of each protein are given, along with what is known about their functions. Two key proteins that are discussed in detail (mentioned earlier in this introduction) include CooA, one of the proteins with a better---understood ligand switch, and human cystathionine β---synthase, or hCBS. This latter protein is important because it is not yet entirely

understood as to how the ligand switch affects the functioning of the protein, and problematic mutations in hCBS can lead to disease states in humans. Learning more about it could lead to understanding more about a protein that can have important biological functions. The second part of this introductory chapter gives more details about the specific spectroscopic characteristics of these proteins, and the differences between them in oxidized and reduced states. Further elaboration about the UV---Visible spectroscopy done for this research can be found in the methodology section.

The second dissertation was written in 2008 by Katherine A. Marvin. Most of the information in the first chapter was similar to the previous dissertation, including the descriptions of the known differences between Type I and Type II proteins, specific protein examples, and various spectroscopic characteristics. Even though the content was analogous to the above information, it was a good reinforcement of the basic characteristics of the proteins being studied in this research, and good background information is essential to working effectively.

Overall the various literature sources gave a great deal of useful insight as to how ligand switches work, as well as specific characteristics of Type II proteins. It was important to learn more about the various states of iron in the heme and how this would be implicated by spectral changes of the protein. Finally, it was crucial to learn more about cytochrome *c*, the protein to be used as a model for this research. Gaining background information about this subject led to the development of a suitable research question to explore for this thesis.

Research Questions to Be Answered

The primary question to be answered during this research project was: what are the necessary mutations that must be made to cytochrome *c* to allow it to function as a Type II protein model? These mutations needed to be identified and carried out in a way that was reproducible in order for this protein to be a valid model. Although progress has been made towards creating this model, there are still inconsistencies that need to be corrected before using it to study the mechanisms of the ligand switch. Once this question is answered and the protein behaves in a manner that is consistent with a Type II protein, the protein can then be used to study how the ligand switch controls the activation of the protein. There were several mutations that were definitely necessary to make a proper protein model. However, after testing of a proper ligand switch mechanism showed that the negative control also underwent a ligand switch when it was not supposed to, it became clear that additional mutations are necessary for the model. Because of this unexpected behavior for the negative control, a new research question had to be developed: what chemical group is the negative control protein using in order to ligand switch, and how can it be eliminated to create a valid model? This question became the primary question for this project, and the exact chemical group used in the ligand switch of the negative control protein remains elusive at this time.

Methodology

PCR

The first stage in the development of a new protein variant is to mutate the DNA that codes for the protein structure. This was done using polymerase chain reaction, or PCR. The DNA of the original protein was used as a template, and DNA primers were designed to mutate specific amino acid sites on the DNA. This newly mutated DNA was "copied" using a thermocycler. A thermocycler has three stages of differing temperatures needed to synthesize many copies of the newly mutated DNA. The stages are denaturing, in which the two strands of DNA are separated, annealing, when the primers can bind to the now separate strands of parent DNA, and elongation, in which the DNA polymerase enzyme builds up the new strands of DNA using the provided DNA building blocks. The enzyme used for elongating the DNA was PfuUltra Polymerase enzyme, which is a high fidelity enzyme, since it "proofreads" as it synthesizes new DNA. The method used involved heating the PCR reaction mixture to a denaturing temperature of 95°C, cooling it to an annealing temperature of 55°C, and then slightly heating it to an elongation temperature of 68°C. This temperature cycle was repeated 20 times, and then the reaction was cooled to 4°C. After the reaction, 1µl of *Dpn*I enzyme was added to the PCR tube, and it was left in a water bath set at 37°C for one hour. This enzyme cleaves any DNA with extra methyl groups, which will only be true of the template DNA. Thus, for the next steps only newly synthesized DNA will be relevant. Afterwards the DNA was stored in the freezer at ---20°C. Before proceeding, the new DNA needed to be tested to ensure that the mutation was successful.

Media Bottle Preparation

The components of the growth plates used for bacterial transformation are stored in two separate bottles and combined immediately before the plates are poured. The first is 2xLC media, which is 2g bacto---tryptone, 1g yeast extract, and 1.2g NaCl. These powders were weighed out and placed in glass bottles, and then 100mL of distilled water was added. The second component of the plate media is 3% agar, a solidifying agent. For this, 3g of bacto---agar was added to a glass bottle, and 100mL of distilled water was added. Both of these bottles were then autoclaved in order to sterilize them. The sterilized components could then be left on the bench indefinitely.

Growth Plate Preparation

The plates were prepared using 100mL of 2xLC media and 100mL of 3% agar. This makes a total of eight plates. The agar was microwaved until it was completely liquefied, and then poured into the 2xLC media. The solution was mixed slightly until it was warm, but not hot. 200µl of the antibiotic ampicillin (a 100mg/mL stock) was added, and the solution was swirled to mix. A 25mL graduated pipet was used to add 25mL of media to each plate. The final antibiotic concentration in the plates was 100µg/mL. The plates were allowed to dry for about 10 minutes, then inverted and placed in a 35.5°C incubator overnight to dry a little further. Afterwards the plates were stored at 4°C until they were used.

Transformation

The DNA from the PCR was incorporated into *E. coli* cells using the heat shock method. DH5α cells (genetically altered to enhance DNA production) that

were already competent to taking up foreign DNA were purchased. First, cells and DNA were combined in a sterile reaction tube. The tubes with the cells and DNA were left to sit on ice for 30 minutes. This gave the DNA time to associate with the cells, which would make it easier to get into the cells. After 30 minutes, the cells were placed in a water bath set at 42°C for 45 seconds. This process creates openings in the cell wall for the foreign DNA to pass through. The cells were then removed from the heat, and 250µl of SOC broth was added to each tube. This is a media that provides many vital nutrients for the cells to promote growth. The tubes were placed in an automated shaker, set at 37°C and 200rpm for one hour. The agitation of the cells continually oxygenates the solution, distributes the nutrients, and allows the cells to stay suspended in the solution so they don't settle to the bottom. After the one---hour growth in the shaker, 130µl of cells from each tube were placed on an LC---Amp growth plate. These plates also have the antibiotic ampicillin, which means that only the ampcillin resistant bacteria from the PCR will be able to grow on the plate. Each transformation tube is split to make 2 LC---Amp plates. The cells were pipetted onto the plate and then spread over the entire surface area using a glass spreader. The spreader was sterilized with ethanol and flame between each plating. The cells were incubated and left to grow overnight at about 35.5°C until individual bacteria colonies were observed.

Liquid Cultures

Once the LC---Amp plates had *E. coli* growth, several individual colonies were chosen for liquid cultures. The cultures were prepared by using a graduated pipet to add 10mL of 2xLC media to a sterile 125mL Erlenmeyer flask. 10µl of 100ng/µl

ampicillin was added to each flask and they were swirled to mix. The colonies for each culture were selected and marked on the plate. An ideal colony was one that was not touching any other colonies and had a relatively uniform size and boundary compared to the other colonies on that particular plate. Using forceps, a sterile toothpick was touched to the marked colony and placed in the flask. The flasks were placed in the shaker at 37°C and 200rpm (revolutions per minute) to grow overnight.

Plasmid Purification

After making liquid cultures, the cells needed to be lysed and the DNA extracted and purified. The flasks were removed from the shaker and placed on ice. Disposable pipets were used to add the cultures to 1.5mL sterile tubes until the tubes were almost full. The tubes were balanced and placed in the microcentrifuge for 1 minute at 11,000rpm. This caused a small cell pellet to form at the bottom of each tube, with the cell---free liquid media at the top. This media was poured off and more of the liquid culture was added to the tube. These steps were repeated until all of the cells had been collected in a pellet at the bottom of the tubes.

At this stage, the Wizard Plus Minipreps DNA Purification System was used to lyse the cells and extract the plasmid. This is a kit with all of the necessary solutions needed for the procedure. First 250µl of Cell Resuspension Solution was added, and the tubes were left to sit for five minutes. The cells were then vortexed until the cell pellets were completely gone, meaning the cells were all resuspended. Next, 250µl of Cell Lysis Solution was added and the tubes were inverted four times to mix, followed by the addition of 10µl of Alkaline Protease solution and four more

inversions. These steps caused the cells to lyse and the proteins released into the solution to be degraded by the alkaline protease enzyme. The tubes were incubated for five minutes at room temperature. 350µl of Neutralization Solution was added and the tubes were again inverted four times. This step caused the remaining protein in the solution to precipitate. The tubes were placed in the microcentrifuge and spun for 10 minutes at maximum speed, 14,800rpm, causing the protein to form a pellet in the bottom of the tube.

For the next stage of the purification process, a special tube was used for each sample. This tube had two components, a spin column, which the plasmid DNA was able to bind to, and a collection tube, in which the rest of the solution would be filtered into. The spin column was inserted into the collection tube. When the cells were done in the microcentrifuge, there was a protein pellet at the bottom and liquid at the top containing the dissolved DNA. The liquid was poured into the spin column, and the pellet was disposed of. The spin column was centrifuged at maximum speed for one minute. The liquid had passed through the spin column and collected in the lower tube. The spin column was removed and the liquid was poured out in the waste, and the spin column was reinserted into the collection tube. At this point the DNA was bound to the filter in the spin column. The column was washed by adding 750µl of Wash Solution, centrifuging for one minute and discarding the flowthrough. The wash step was repeated, this time with 250µl of Wash Solution. After the second wash flowthrough had been discarded, the spin column and collection tube were centrifuged for two minutes at maximum speed in order to ensure that there was no more wash solution associated with the upper

spin column. The spin column was transferred to a sterile 1.5mL tube for each sample. 50µl of nuclease---free water was added to each spin column, and the tubes sat for one minute to allow the DNA on the filter to dissolve. The spin column and tube were then centrifuged for one minute at maximum speed. This step caused the plasmid to flow through the spin column and collect in the 1.5mL tube. The spin column was removed from the tube, and the plasmids were stored in the freezer at ---20°C.

UV--Visible Spectroscopy: Plasmid Analysis

In order to determine the concentrations of purified plasmids, UV---Vis spectroscopy was used. DNA has a distinct absorbance pattern, with a peak at about 260nm and relatively no absorbance at 350nm. Measuring the absorbance at 350nm is a way to subtract the background noise from the spectrum and obtain a more accurate concentration. The spectrum was obtained with a minimum wavelength of 200nm and a maximum of 400nm. Before running the plasmids, the instrument baseline was zeroed using a microcuvette with 80µl of distilled water. This blank is run a second time to ensure there are no peaks in the spectrum (i.e., the baseline is properly zeroed). The samples were then diluted 20---fold, meaning 4µl of the plasmid was added to 76µl of distilled water. The samples were flicked to mix, then spun down for about 10 seconds in a microcentrifuge. The samples were then placed in the same microcuvette used to run the blank and the absorbance values at 260nm and 350nm were recorded. Before adding each sample, it was important to be sure that the entire previous sample had been removed from the cuvette to ensure a more accurate concentration reading. These concentrations were then

added to a spreadsheet with the concentration values for all of the DNA mutants. The spreadsheet was set up to automatically calculate the concentration of the plasmid DNA sample using the absorbance at 260nm and 350nm.

Sequence Analysis

The purified DNA was diluted to a specific concentration and sent to Iowa State University to be sequenced. The sequence results were sent through an online account, and they were interpreted using the viewing program called Chromas and an online gene database and alignment program called BLAST. By comparing the modified DNA to the original DNA in the database, the success of the mutation was determined. If the new amino acid sequence was incorporated to the template DNA, the next set of experiments could proceed.

Protein Purification

Successfully mutated DNA was then combined with a different strain of *E. coli*, BL21(DE3), which is used for expressing protein. The transformation procedure is the same as it is for the other strain of *E. coli*, with the cells sitting on ice for 30 minutes, followed by heat shock, the addition of SOC media, and an hour---long incubation on the shaker. The cells were then added to two plates per sample and put in the incubator overnight. The liquid cultures prepared for these samples were done on a much larger scale. Instead of 10mL volumes of media, a new kind of flask was used. These large plastic flasks were called Fernbach flasks, and they were used for large liquid cultures. For each Fernbach flask, 2xYT media was prepared directly in the flask. The 2xYT media consisted of 16g tryptone, 10g yeast extract, 5g of NaCl, and 1000mL of deionized water. This was then autoclaved for 20 minutes to

sterilize the media. The flasks were labeled and 1.5mL of 100ng/µl ampicillin was added to each flask. Instead of touching a single colony to a toothpick to inoculate the flasks, the entire plate of colonies was added. This was done by suspending the colonies on the plate using 3mL of LC media and a glass spreader. The spreader was sterilized between each sample using a flame and ethanol. The liquid on the plate was then pipetted off and placed in the flasks, and the flasks were put in a large shaker set at 200rpm and 37°C, overnight. If the samples created a large amount of foam, some antifoam solution was added to make the protein samples easier to pour. The protein was poured into a Nalgene centrifuge bottle, about ¾ of the way full, and it was centrifuged for 10 minutes at 5,000rpm and 4°C. The samples were then removed from the centrifuge and the liquid was poured into a waste flask. The process was repeated until all of the culture flasks were empty. This left behind a cell pellet containing the protein of interest.

Once the cells pellets were isolated, the cells needed to be lysed. This is essentially breaking apart the cells in order to have access to the protein of interest. The components for a cell lysis were 10x BugBuster, Tris lysis buffer, and Benzonase nuclease. The BugBuster is a detergent---like solution that breaks the cells apart. These three components were mixed in volumes that corresponded to the size of the cell pellet, and the pellets were placed on an orbital shaker to agitate them until there was no solid left in the bottle. This process typically took about one hour. An additional option to ensure complete lysis was sonification, the use of intense sound waves that further broke the cells apart. However, this proved to have little additional effect on the overall lysis process and was not used for every purification.

After cell lysis the pellets were spun down in the centrifuge for 30 minutes at 10,000rpm and 4°C to remove the insoluble material from the sample, which includes cell membranes and any associated proteins.

After removing the bottles from the centrifuge, the protein of interest was obviously apparent in the liquid above the pellets, as the liquid was red. The supernatant was poured out into graduated cylinders in order to measure the volume. Once the volume was known, the liquid was transferred into an Erlenmeyer flask, and placed on a stir plate. Ammonium sulfate salt was added slowly to a total amount of 0.26g for every mL of protein. The mix was left on the stir plate and then moved into the refrigerator overnight. After one night, the flasks were taken out of the refrigerator and poured back into Nalgene bottles which were balanced and centrifuged for 30 minutes at 10,000rpm and 4°C. This created a small pellet, with the protein of interest being in the supernatant. While the supernatant was brighter pink, the small pellet containing unwanted proteins was generally white to pale pink.

The centrifuged liquid was then poured into dialysis tubing, which is used to filter out only certain molecules. A knot is tied at one end of the tubing and the protein is poured in the other. The top is twisted and clamped shut, and the dialysis bag is placed in a large jar of dialysis buffer. This is placed on a stir plate and stored in the cold room overnight. Because only select molecules can pass through the dialysis membrane and into the buffer, the protein stayed trapped in the tubing, while small molecules crossed the membrane and flowed into the buffer. This method was used to drastically reduce the concentration of ammonium sulfate in

the sample. After one night, the protein in the dialysis bag was removed from one jar of dialysis buffer and placed in a second, fresh buffer for further dialysis. It was again placed on a stir plate in the cold room (4°C) and left overnight.

Once the protein had been in dialysis buffer for two nights, it was centrifuged for 30 minutes at 10,000rpm and 4°C. Again, the protein of interest stayed in the supernatant, which was bright red. At that point the samples were ready for ion---exchange column chromatography. This is a method that can separate one protein from the remaining cellular components based on the charge on the protein. CM (carboxymethyl) Sepharose resin is made up of tiny beads, and different proteins will associate with the beads for different lengths of time depending on their charge. A buffer is rinsed through the column, and protein will begin to elute based on the salt concentration of the buffer, as a high concentration of ions in the solution will disrupt the interaction between the protein and the resin. The concentration of the buffer is slowly increased, and eventually it is high enough to elute the protein of interest, which has a distinct red color.

The supernatant from the final centrifuge run was poured into a 1L flask containing CM---Sepharose resin that was previously equilibrated in CM---Low buffer. CM---Low buffer is a low salt buffer that will allow the cytochrome *c* protein to stick to the resin. The protein---resin mixture was stored in the refrigerator for about one hour in order to let the protein associate with the beads. The resin was then poured into a glass column and rinsed with CM low buffer for approximately one hour to wash off any proteins that did not stick to the resin.

Once the chromatography column had been washed, a gradient mixer was set up. This device consisted of two tubes, which had a small opening between them. One tube had CM---Low buffer, and the other had CM---High buffer. The CM---High buffer contains a high concentration of sodium chloride. The tube with the CM---Low buffer had an additional opening that was connected to the chromatograpy column. As the CM---Low buffer began to drip into the column, the reservoir holding the CM---Low buffer would be depleted, and in order to maintain the volume balance in both reservoirs, the CM---High buffer would automatically start to leak into the CM---Low reservoir. In this way, the concentration of the buffer was gradually increased. The buffer that flowed through the column was collected in a waste beaker, until the distinct color of the protein was observed to drip out the bottom. At this time, the waste beaker was replaced with a collection beaker, and the protein was collected until no more of the protein color could be observed on the column. At this time, the waste beaker was replaced and the concentration gradient was allowed to run its course. This ensured that any remaining impurities from the sample would be washed off with the CM---High buffer and the column resin could be reused. The column was then re---equilibrated with CM---Low buffer and the resin was unpacked from the column into a 1L flask and stored in the refrigerator at 4°C.

Once the protein had undergone purification by chromatography, the final step in the process was protein concentration. This was done using a centrifuge and Centricon concentrators, which are special centrifuge tubes with two compartments: an inner chamber and an outer chamber. The compartments were separated with a filter that allowed buffer to pass through to the inner chamber,

while the larger protein molecules would be stuck in the outer chamber. The tubes were centrifuged for 30 minutes at 3,000rpm and 4°C. The liquid in the inner chamber was inspected to make sure it was colorless and no protein had escaped through the filter, then it was disposed of. This process was repeated until the entire protein sample had been concentrated. Once this had been completed, the protein was diluted with HPLC A buffer and centrifuged. This process is referred to as a buffer exchange, and it is done to ensure that all of the CM---High buffer had been washed away, because the high salt concentration in the buffer can damage the protein over longer time periods, or it can interfere with subsequent experiments. Once the protein had been re---concentrated after the buffer exchange, the proteins were divided into 1mL aliquots in 1.5mL tubes. The tubes were flash---frozen using liquid nitrogen, then stored in the freezer at ---80°C.

Dialysis Buffer Preparation

In order to prepare the buffer solution for dialysis, the following components were weighed and put in a large glass jar (enough to hold about 2750mL of solution): 1.72g $NaH_2PO_4 \bullet H_2O$ (monobasic), 3.11g Na_2HPO_4 (dibasic), and 1.08g Na_2EDTA. The jar was placed on a stir plate with a magnetic stir bar, and 2600mL of deionized water was added. Once all of the solid was dissolved the pH was adjusted to about 7.2 using either NaOH or HCl. The volume of the acid/base solutions used to adjust the pH was added to the total volume, and the rest of the water was added until the total solution volume was 2744mL. The final pH was taken and slight adjustments were made with acid and base to ensure the solution was at about pH 7.2. The buffer was stored in the cold room at 4°C until used for the protein

purification. Directly before using the dialysis buffer, 385µl of beta---mercaptoethanol, or BME, was added to the solution. It is best to add BME directly before using the buffer because it will be more effective, as it reacts with oxygen over time.

HPLC Buffer A Preparation

The HPLC Buffer is used in the protein purification process. To prepare a one liter volume, the following components were combined: 2.97g $NaH_2PO_4 \bullet H_2O$ (monobasic) and 4.04g Na_2HPO_4 (dibasic). The powders were weighed out and placed in a 2 liter beaker with a magnetic stir bar on a stir plate. 900mL of distilled water was added and the powders were dissolved. The pH was adjusted to 7.0 using a pH probe and solutions of NaOH and HCl. Once the pH was correct, the solution was brought up to a total volume of 1 liter. The pH was checked one final time and slight adjustments were made if necessary to maintain a pH of 7. The solution was stored in the cold room at 4°C.

UV--Visible Spectroscopy: Protein Analysis

In order to analyze the final protein product, UV---Vis spectroscopy was used. The iron in the heme of cytochrome *c* is found in either the +2 or +3 oxidation state, and each of these states absorbs different wavelengths of light. Because the oxidation state of the iron is an indicator as to whether the ligand switch has occurred, this spectroscopic analysis was a good indicator of how specific mutations in protein structure affected overall protein function.

SDS--PAGE

Denaturing polyacrylamide gel electrophoresis (SDS---PAGE) was used in order to separate proteins of different sizes. When a cell pellet was produced with a brown or tan color not normally associated with cytochrome *c* expression, the proteins in the cell pellet were denatured and run on a gel along with purified cytochrome *c* as a control. If the band associated with cytochrome *c* matched a band of protein in the newly harvested cells, it showed that the new sample successfully expressed the protein of interest.

There were several components that needed to be prepared for an SDS---PAGE. First was the Sample Buffer. This involved mixing 3.55mL of water, 1.25mL of 0.5M Tris•HCl (pH 6.8), 2.5mL of glycerol, 2.0mL of 10% SDS, and 0.1mg of bromophenol blue. The next component that needed to be prepared was the 15% Separating Gel. This was the main gel component that the protein would be separated in. The following components were added: 1.2mL of distilled, de---ionized water, 2.5mL of 30% acrylamide/0.8% bis---acrylamide solution, 1.25mL of buffer (1.5M Tris•HCl, pH 8.8), and 0.05mL of 10% SDS. The final component to be prepared was the 6% Stacking Gel. This gel was poured on top of the Separating Gel. This is the gel that the samples are actually added to, and it allows the protein to form a uniform band before actually hitting the separating gel. To prepare it the following components were combined: 0.5mL 30% acrylamide/0.8% bis---acrylamide solution, 0.65mL of buffer (0.5M Tris•HCl, pH 6.8), 0.025mL 10% SDS, and 1.325mL water.

The gel plates were assembled. These were two thin glass plates that the gels would be poured into and solidified in. The first gel to be added was the Separating Gel. Before adding it to the gel plates, 30µl of 1% (w/v) ammonium persulfate, or APS, and 10µl of TEMED were added to the gel solution. These caused the gel to begin to polymerize, which would allow it to eventually retain a solid shape. Immediately after adding the APS and TEMED, a glass pipet was used to suck up the gel mixture and put it in between the two glass plates. The gel sat for a few minutes, and a layer of water was added to the top of the plates to help even the top of the gel out. The water was poured out, and the same process was used to add the Stacking Gel, this time using 13µl of 1% (w/v) APS and 3µl of TEMED. A comb was inserted at the top of the gel plates to form the wells for the protein samples, and the gel was allowed to solidify.

The cell pellets were dissolved in sample buffer and the denatured protein samples were heated and spun down in order to prepare them for the gel. An electrophoresis tank was set up, and the gel was placed inside. The comb was removed and the inner chamber was filled with 1x Running Buffer. Each well was rinsed with about 50µl of running buffer using a syringe. The syringe was then used to load the samples, rinsing with water between each one. Once the samples were loaded, the remaining running buffer was poured into the tank and the lid was placed on. The lid had leads that were plugged into the power source, matching like colors. The gel was run at 200V and 100mA for about 45 minutes. Once the bromophenol blue dye was close to running off the bottom of the gel, the power was turned off. The gel was removed from the tank and the glass plates were pulled

apart. A small razor was used, first to remove the stacking gel, and then to cut a corner in the gel in order to identify when the gel was in the proper orientation.

The next step was to stain the gel so the protein bands could be identified. The gel was first rinsed with a small amount of tap deionized water to remove any adhering buffer. A flat glass bowl was used to pour in a layer of blue stain around 0.5---1 inches deep. It was microwaved until hot. The gel was placed in the stain for about 40 minutes, then removed and rinsed with water. A 10% acetic acid destain solution was microwaved until warm, and the gel was placed in it to de---stain. Kim---wipes were placed in the container and weighed down in order to absorb the dye. The gel was placed on a slow moving shaker overnight. This process removed excess dye from the gel, which left only the dye associated with the protein bands. Once the gel had been de---stained, it was rinsed with de---ionized water, then placed in fresh water on the slow moving shaker for about one hour. The water was removed and replaced with a 30% methanol solution for an additional hour on the shaker to slightly dehydrate the gel. The gel was placed between two sheets of drying film on a drying frame, which was clamped together and left overnight. The dried gel was then cut out of the frame and placed under a heavy object to help flatten of the gel. The gel was then ready to be analyzed and stored.

Experimental Timeline

DNA Mutations

Before beginning the overall procedure for this research, it was important to understand the nomenclature for the protein samples. The proteins are given a variety of mutations at specific places in the amino acid sequence. A protein with no

mutations is given the designation WT, which stands for wild---type. The proteins used for this project were given several standard mutations (K72A and C102S), and were therefore given the designation of WT*. For each mutation that was made, the letter corresponding to new amino acid, as well as the position in the amino acid chain, was added to the name. For example a protein with the name WT*/A73 was mutated to have an alanine in the 73rd position of the protein chain, and a protein named WT*/A73/C80 had a mutation to alanine at the 73rd position and a cysteine at the 80th position.

The first stage of the experiment consisted of mutating several strains of DNA to have a cysteine at position 80 of the amino acid chain. In cytochrome *c*, the 80th amino acid, which is normally methionine, is attached to the heme, so it is important that this matches real Type II proteins for the model to be valid. Two different strains of parent DNA were used for this initial experiment: WT*/H73 and WT*/A73. The primers used were K79/M80C and K79/M80Cr. The M80C indicates that the parent DNA has a methionine in the 80th position, and it will be mutated to a cysteine. The second primer has a lowercase "r," indicating that it is the reverse primer for the mutation. Both a forward and reverse primer are necessary for the PCR reaction. The parent DNA was diluted to a concentration of 100ng/µl, and the primers were diluted to concentrations of 125ng/µl. The PCR mix listed on the following page was created:

Table 2: PCR Mix

Component	Amount (µl)
Sterile Water	41
10x Pfu Buffer	5
10mM dNTPs	1
Parent DNA	1
Forward Primer	1
Reverse Primer	1
Pfu Ultra Polymerase Enzyme	1

Before mixing the reaction components, the Pfu buffer, dNTPs, DNA, and primers needed to be thawed and spun down in a microcentrifuge to be mixed. After the Pfu Ultra Polymerase enzyme was added, the solution was flicked to mix, then spun in the microcentrifuge for about one minute to get rid of any air bubbles that had formed in the tube. The total reaction volume was 51µl, and the "Cherney" mutagenesis method was used on the thermocycler. Once the PCR was complete, 1µl of *Dpn*1 endonuclease was added and the sample was placed in a 37°C water bath for one hour. This digested the parent DNA. The samples from the reaction were labeled WT*/A73/C80 and WT*/H73/C80.

After the DNA had been mutated in the PCR, it was necessary to do a transformation to incorporate the new DNA into cells. The cells used was a 50µl aliquot of the strain DH5α. In addition to the WT*/A73/C80 and WT*/H73/C80 from the PCR, three additional mutants were transformed: WT*, WT*/A73/A79, and WT*/A73/H79. These three strains had been mutated previously and were stored in the freezer at ---20°C. For this transformation, 10µl of cells were combined with 3µl of DNA in five separate 1.5mL sterile tubes. The above heat shock transformation procedure was followed, and the cells were incubated overnight. WT*/A73/A79 and

WT*/A73/H79 both grew over 100 colonies per plate, but the WT* and the two mutants from the PCR did not grow any colonies on either plate.

Because the transformation was not successful, the PCR was repeated for WT*/A73/C80 and WT*/H73/C80. New dilutions of the parent DNA and primers were prepared, and new buffer was used as well. For the actual reaction, the annealing temperature was lowered from 55°C to 53°C, because in some cases a slightly colder annealing temperature makes it easier for the primers to bind to the DNA. In addition to repeating the PCR, the original PCR mix from the first experiment was used to repeat the transformation for these two mutants, as well as WT*. 16.6µl of cells and 5µl of DNA were used for each sample. After incubating these samples overnight, colonies grew on all four WT*/A73/C80 and WT*/H73/C80 plates. No colonies grew on the WT* plates, but the original transformation plates for the WT* mutant had been left out at room temperature in an attempt to grow colonies, and one colony had grown on one plate. Because the second transformations were successful, the DNA from the second PCR was not needed, and it was stored at ---20°C.

The following 10mL liquid cultures were prepared for the transformed cells:

Table 3: Liquid Culture Information

Mutant	Number of Cultures
WT*/A73/A79	1
WT*/A73/H79	1
WT*/A73/C80	3
WT*/H73/C80	3
WT*	1

Extra liquid cultures were made for the cells containing DNA that had just been mutated with PCR, because the PCR reaction does not have a 100% success rate. Some of the cells will take in new DNA with a sequence equivalent to the original parent DNA, so taking several liquid cultures increases the chances that the correct DNA will be found. Because the WT* plate had only one colony, the tip of a sterile toothpick was used to spread the edge of the colony across the plate, in an attempt to grow slightly more bacteria. This toothpick was then used in the liquid culture. The cultures were prepared using the procedure outlined above and left to shake overnight. The next day, the liquid, which had initially been clear, had a cloudy appearance that indicated the cultures grew successfully.

After shaking overnight, the cells were ready for plasmid purification. This procedure involved lysing the cells and extracting and purifying the plasmid. The cultures were removed from the shaker and placed on ice. They were spun down in a centrifuge so the cells were collected in a small pellet and the supernatant was poured off. The plasmids were purified using the Wizard Plus SV Minipreps DNA Purification System. The full purification procedure is described in detail above. Once the plasmids were purified, they were diluted 20---fold in order to determine their concentrations by UV---Visible spectroscopy. These concentrations were added to a spreadsheet database that contained plasmid information for all mutants.

While preparing the WT*/A73/C80 and WT*/H73/C80 mutants, two mutants that had been in storage at ---20°C were prepared to be sent to Iowa State University to be sequenced. These samples were WT*/A79/C80 and WT*/H79/C80, with three samples for each mutant. The samples were prepared by making 5µl

solutions with a concentration of 250ng/µl. The dilutions were done with sterile water. The tubes were flicked to mix, spun down in the centrifuge, and wrapped in Parafilm in order to ensure that the tubes remained sealed during shipment.

After the DNA had been sequenced, the information was sent to an online account and available for downloading and analyzing. The mutation of interest was a change in the three base pairs that code for the 80th amino acid in the protein. The expected change in base pairs was ATG to TGC. Sequence documents were generated for each sample, and the computer program BLAST was used to compare the sample to the original DNA so the success of the mutation could be identified. As long as one of the three samples had an intact plasmid with the correct mutation, the PCR was considered a success. WT*/A79/C80 had one sample with the correct mutation, but all three of the samples for WT*/H79/C80 were incorrect, meaning the sequence analysis showed that the sample contained only the original parent DNA, and did not have the cysteine mutation at the 80th amino acid position.

In order to attempt to get a sample with the correct mutation, three more liquid cultures were started for WT*/H79/C80 from the plates that had been stored in the refrigerator at 4°C. These cultures were then harvested, the DNA was purified and the DNA concentration was found with UV---Vis spectroscopy. These samples were then prepared for sequencing along with the six WT*/A73/C80 and WT*/H73/C80 samples.

Once the sequence results were analyzed, it was found that two out of the three WT*/A73/C80 plasmids were correct, but none of the WT*/H73/C80 or WT*/H79/C80 mutations had worked. Because the WT*/H79/C80 mutant had

failed six times, the PCR was repeated. In this reaction, the annealing temperature was lowered from 55°C to 53°C. After the reaction in the thermocycler, the *Dpn*1 enzyme was added and the tube was placed in the water bath for 2 hours. A second PCR had already been run for the WT*/H73/C80 mutant. Both of these samples were transformed, adding 25µl of cells to 7µl of DNA in 1.5mL sterile tubes. The above transformation procedure was followed. After incubating overnight, WT*/H79/C80 had many colonies; WT*/H73/C80 had a total of three colonies.

Three liquid cultures were made for each cell type. Since the WT*/H73/C80 mutant only had three colonies to begin with, each colony was streaked across the plate in an attempt to get a little more cell growth. These plates were left out at room temperature overnight, while the other two plates were wrapped with Parafilm and stored at 4°C. Once the liquid cultures had been incubated, the plasmids were purified, the DNA concentrations were measured using spectroscopy, and the samples were diluted and prepared for sequencing.

The samples were sent to Iowa State, and the sequences were downloaded and analyzed. WT*/H73/C80 had no correct sequence; it was all parent DNA, but for WT*/H79/C80, one sample had the correctly mutated sequence. The PCR was repeated for WT*/H73/C80 until the correct mutation was obtained. At this time another PCR reaction was set up to add the C80 mutation to two additional DNA mutants: WT* and WT*/A73/H79. These samples were prepared but did not need to be sequenced at the time, so they were stored at ---20°C.

Protein Purification

Before working with the proteins that had the C80 mutation, several other proteins were purified to be used as controls. These variants had been previously transformed and stored at ---80°C as protein pellets. The following table gives the names of the variants used for purification, along with the mass of the protein pellet. BL21(DE3) is the strain of *E. coli* cells used for the transformation process.

Table 4: Control Protein Pellets

Pellet Name	Mass(g)
BL21 WT*/H73 1	9.365
BL21 WT*/H73 2	9.415
BL21 WT*/H79 1	7.67
BL21 WT*/H79 2	8.31
BL21 WT*/A79 1	7.479
BL21 WT*/A79 2	7.97
BL21 WT*/A73 1	7.31
BL21 WT*/A73 2	7.87

Because the pellets had been stored at ---80°C, they needed to undergo several freeze/thaw cycles before beginning the purification process. For the first cycle, the pellets were placed in the refrigerator, but the complete thaw took almost seven hours. So from that point on the freeze/thaw cycles were done by placing the bottles with the cell pellets in them in a large beaker of cool water. This was placed on a stir plate until the pellets were runny, then the pellets were placed back at ---80°C to refreeze. Typically three freeze/thaws were done for each pellet. The pellets were then ready for purification.

Only four samples were chosen for purification at first: WT*/H73 1 and 2, as well as WT*/H79 1 and 2. The full details of the purification process can be found in the methodology section. After cell lysis, the tubes of cell lysate were centrifuged.

The volumes of cell---free lysate were combined, so there was only one WT*/H73 protein sample and one WT*/H79 protein sample. The volumes of supernatant for the WT*/H73 and WT*/H79 variants were 97.5mL and 79.8mL, respectively. The samples underwent the ammonium sulfate addition and two nights of dialysis before they were ready for the column chromatography. At this point the supernatant was a vivid pink in color.

Upon the addition of the column and the initial wash with CM---Low buffer, it was found that both proteins began to elute, meaning the concentration of the CM---Low buffer was already too high. A 1:1 dilution was made of the buffer in water, so the concentration could be low enough to rinse the column without eluting the proteins. It is undesirable for the proteins to elute immediately, because there would be no separation of the impurities that are bound more weakly to the column. WT*/H73 was a pale red---orange color, and WT*/H79 was pale red. The proteins were then concentrated, and both variants turned a deep red color.

For each protein, 4µl was taken and diluted in 76µl of HPLCA buffer in order to obtain a UV---vis spectrum. The spectra showed that the total protein yield for WT*/H73 was 53.3mg and the yield for WT*/H79 was 27.0mg. The samples were then divided up into aliquots, frozen in liquid nitrogen, and stored in the freezer at ---80°C. Following the purification of these two samples, the remaining proteins were purified.

At this time, successful PCR had created several C80 mutants, and four were chosen for purification: WT*/C80, WT*/A73/H79/C80, WT*/H73/C80, and WT*/H79/C80. The DNA was transformed, and all variants except WT*/H79 had

colonies. The other three variants were added to the large liquid cultures, two for each plate (a total of six cultures) and incubated overnight. The next day, the two cultures for each variant were combined and centrifuged until only a solid protein pellet was left. The final mass of each pellet was measured. WT*/C80 and WT*/H73/C80 had darker brown pellets with a slight red tint, while WT*/A73/H79/C80 had lighter brown pellets. The pellets were stored at ---80°C.

Two variants, WT*/C80 and WT*/H73/C80 were chosen to be purified because they had a promising darker color. The freeze/thaw cycles were done by thawing with a large beaker full of water on a stir plate, followed by a freeze in liquid nitrogen for about one minute. The pellets were then lysed and centrifuged. The full methodology for the protein purification process can be found above. The total volumes for the cell---free lysate of WT*/C80 and WT*/H73/C80 were 90.9mL and 88.1mL, respectively. This was followed by ammonium sulfate addition, dialysis, and column chromatography. The protein that had been purified previously had a pinker color to it, but because of the addition of the C80 mutation, these variants were a darker brown in color with a slightly orange tint. The proteins were concentrated, divided into aliquots, and placed in the fridge.

Protein Analysis

Once several proteins with the C80 mutation had been purified, it was possible to test their behavior under various conditions using UV---Visible spectroscopy. There are two distinct spectra that can be obtained: one occurs when the iron in the heme is in an oxidized state, the other when the iron is in a reduced state. The change from an oxidized to reduced state is also indicative of a ligand

switch occurring, so the changes in the spectra from oxidized to reduced was the method used to determine whether or not the protein was undergoing the ligand switch when exposed to various environments.

The first factor to be tested was the effect of pH on the protein's ability to ligand switch. Three different buffers were prepared: a MOPS buffer at pH 7.0, and two Boric acid buffers, one at pH 9.0 and one at pH 10.0. The first protein to be tested was WT*/H73/C80, diluted 15---fold by adding 16µl of the protein to 228µl of the buffer for a total volume of 244µl. One dilution was made for each buffer. The UV---Vis spectrophotometer was blanked using a water sample, and each buffer was then run without any protein to be sure that there would be no significant peaks caused by the buffer itself.

The samples were then run. Each sample was in an oxidized state for the initial sample run (Figure 4). The next step was to see if a reducing agent could be added to induce the ligand swith. A small amount of sodium dithionite, or DTH, was added to each sample and a second spectrum was taken immediately after the reducing agent was added. This step was done quickly in case the oxygen in the air was enough to reoxidize the sample before an accurate spectrum could be taken. Initially a rubber septum and nitrogen line was used in an attempt to clear oxygen from the air space in the cuvette, but it was determined that this step was unnecessary as long as the second spectrum was taken immediately after the DTH was added. All three samples were reduced, showing a successful ligand switch.

Next, the WT*/C80 samples were prepared with the same 15---fold dilution and final sample volume. The spectra were taken, and a small amount of DTH was

added (Figure 5). All three samples were reduced. This was unexpected. The WT*/C80 was supposed to act as a negative control at this stage in the experiment, because it did not have an H73 or H79 mutation. Without a mutation in these spots of the protein, it was expected that the protein would not be reduced, because it would have nothing to ligand switch with. The fact that the WT*/C80 protein was able to be reduced meant that it had in fact undergone a ligand switch, even though there shouldn't have been any nearby amino acids for the switch to occur. This variant was tested further by using a micropipette to blow air into the samples. A third spectrum was taken for each, and all three had been re---oxidized. The reversibility of the ligand switch was tested even further by adding more DTH to the pH10.0 sample. A fourth spectrum showed that the protein was able to reduce for a second time. The line in this spectrum had shifted up slightly, possibly from the protein beginning to denature.

The proteins were then tested using more acidic buffers. At acidic pH some amino acid side chains that may otherwise act as ligands would be protonated. If some of the proteins could not be reduced at acidic pH it would elucidate more about the amino acids acting as heme ligands. Three buffers were made using citric acid, one at pH 4.0, one at pH 5.0, and one at pH 6.0. Slightly larger samples were used this time: 24µl of protein to 342µl of buffer for a total sample volume of 366µl. First the spectra were taken for WT*/H73/C80, with reductions using DTH attempted at each pH. Each sample could be reduced. Reoxidation was attempted at pH 4.0, but the sample became cloudy, and it was likely that the protein had denatured. Next the spectra were taken for WT*/C80. Again, each sample could be

reduced. The sample at pH 6.0 was reoxidized using air, and re---reduced with more DTH, again demonstrating the reversibility of the ligand switch. The reoxidation was attempted at pH 4.0 as well, but the protein appeared to denature.

After testing the variants with the C80 mutations, two variants without this mutation were thawed from the ---80°C storage: WT*/A73 and WT*/H73. These samples were diluted 15---fold to the 366µl sample volume using the buffers at pH 7.0, 9.0, and 10.0. These samples all started out reduced, and all were able to be oxidized with a few crystals of the oxidizing agent $K_3Fe(CN)_6$.

Protein Expression

Because WT*/A73/H79/C80 had light brown pellets, it was likely that it was not expressing much cytochrome *c*, which has a red color to it. Several methods were tested in order to determine if there was a better way to get more protein expression out of the cells. A transformation was done for this variant, along with WT*/H79/C80, WT*/A73/A79/C80, WT*/A73/C80, and WT*/A79/C80.

The first test for better protein expression was done with a small sample culture containing 20mL of 2xYT media. Instead of using one colony per culture, a toothpick was used to scrape a dense area of colonies off the plate so more cells were added to the culture. The cultures were put in the shaker overnight. The next day, the cultures were poured into 50mL plastic tubes and centrifuged for 10 minutes at 5,000rpm and 4°C. The pellets were removed from the centrifuge and the media was poured out. All of the pellets were a very pale brown, with no obvious red color.

The next attempt at better protein expression involved the addition of iron to the cultures, which has been known to aid in the expression of certain heme proteins. A ferric stock solution was prepared by adding 0.541g of ferric ammonium citrate and 0.541g of citric acid to 50mL of water. The liquid cultures were prepared for each sample by adding 10mL of 2xYT, 10µl of ampicillin, and 10µl of the ferric stock solution. A dense clump of cells was used to inoculate each culture. In addition to the iron, the cells were incubated overnight at a lower temperature, 28°C. The next day, the cells were centrifuged and the media was poured out, but the cell pellets were still a very pale brown.

The third attempt at improving expression involved an even better media: 10mL of Terrific Broth, 10µl of ampicillin, and 10µl of the ferric stock solution. The temperature of the shaker was set at 25°C, but the actual running temperature was closer to 27---28°C. These cultures were centrifuged, and even though there was still no red tint, the pellets were a slightly darker brown than before.

Once some darker pellets had been produced, an SDS---PAGE gel was run in order to see if any cytochrome *c* was actually in the pellet. The samples used were WT*/H73, WT*/A73 (as controls, because both of these samples were known to have cytochrome *c*), WT*/C80, WT*/H73/C80, WT*/A79/C80, and WT*/H79/C80. The final two samples were not purified protein, but small clumps of the cell pellet from the Terrific Broth liquid cultures. The purified samples were prepared by adding 20µl of sample to 20µl of running buffer. These were mixed, and the samples turned a dark blue. Because the pellets were harder to dissolve for the gel, between 600---700µl of sample buffer was added to the small pellet, with vigorous vortexing to

mix. There was a small amount of pellet that didn't dissolve, but there was enough to run the gel.

The full procedure for running an SDS---PAGE can be found in the methodology section. Between 10 and 20µl of sample was added to each well. There was enough room on the gel for several samples to have two wells, in which case two different volumes were used to see if the sample size had an effect on the results of the gel. Once the gel was stained, the bands could be analyzed to determine which samples had a good amount of cytochrome *c*. It was found that the unpurified cell pellets were mainly impurities, and did not have a detectable amount of protein compared to the other control samples.

Determination of the Unknown Ligand

Because it was crucial to have a protein that could act as a negative control and not undergo a ligand switch, it was essential to determine what the WT*/C80 protein was using as a ligand when the reducing agent was added. The first step was to do additional PCR on the WT*/C80 and WT*/H73/C80 variants. Three additional primers were ordered to mutate the DNA. The first two, H33N and H39Q, were prepared in order to remove two additional histidines from the amino acid sequence. This amino acid is the main one used for ligand switching in this model, therefore removing the additional histidines may prevent the control protein from ligand switching if they are in fact the cause of the protein's ability to switch when the reducing agent is added. The primer H33N will change the histidine at the 33rd position to an asparagine, and the H39Q primer will change the 39th histidine to a glutamine. The third primer, N52I is for adding a stabilizing mutation to the protein.

It will change the asparagine in the 52nd position to an isoleucine, which adds more durability to the overall protein structure. Once these primers were ordered, the concentrations were found using UV---Vis spectroscopy.

The first mutation to be added was H39Q, making the new mutants WT*/Q39/C80 and WT*/Q39/H73/C80. This primer was added to the PCR mix, the cells were transformed with the PCR DNA, and liquid cultures of the cells were started. The plasmids were purified and their concentrations were measured using UV---Vis spectroscopy. The samples were then prepared for sequencing and sent to Iowa State University. The results were analyzed, and it was found that one of the samples for WT*/Q39/C80 was correct, while neither of the WT*/Q39/H73/C80 samples were correct. Two more liquid cultures were prepared for this mutant. The plasmids were purified, the concentration was found using UV---Vis, and the samples were stored at ---20°C until they would be sent for sequencing.

Two new PCR were started, one to add the next mutation to WT*/Q39/C80, making it WT*/N33/Q39/C80, and one to add a stabilizing mutation to WT*/H79/C80, making it WT*/I52/H79/C80. Once the PCR was complete, cells were transformed with the PCR DNA, and two liquid cultures were made from the cells containing each mutant. The plasmid DNA was purified and the concentrations were found using UV---Vis. These two mutants, along with WT*/Q39/H73/C80, were prepared for sequencing and sent to Iowa State. The results were analyzed, and at least one sample had the correct mutation for each mutant.

After the successful mutations for the previous three samples, two additional PCR's were set up. One was to add the N33 mutation to WT*/Q39/H73/C80, and

one was to add the stabilizing I52 mutation to WT*/N33/Q39/C80. The new mutant DNA was prepared by PCR, multiplied in DH5α cells, extracted, and prepared for sequencing. The N33 mutation was unsuccessful, but the WT*/N33/Q39/I52/C80 mutant worked. Two more liquid cultures were prepared for the failed mutant, and this time the sequencing showed that the mutation had been successful, creating WT*/N33/Q39/H73/C80.

At this point several different mutants had been created. These mutants were transformed into BL21(DE3) cells and the cell colonies were used to make either large Fernbach flask cultures or smaller 10mL liquid cultures. Two additional SDS---PAGE gels were run using small chunks of the pellets from the liquid cultures, in order to determine which samples would be the best candidates for a protein purification. It was decided that the red/brown WT*/I52/H79/C80 and the pale WT*/N33/Q39/H73/C80 would be used.

The cells were lysed using the BugBuster method, and the cells were resuspended. Instead of doing a full protein purification at this point, the suspended cells were diluted and a UV---Vis spectrum was taken for each sample. Even though there was considerable noise in the spectra due to the impurities of the samples, it was determined that the darker pellet did have a peak indicative of the cytochrome *c* protein, while the paler pellet had almost no peak. The lysis was repeated for a third pellet, WT*/N33/Q39/I52/C80, which had an intermediate brown color. The spectrum showed a smaller peak, but one that was noticeable enough to assume that a protein purification would yield enough protein that the variants could be

tested in order to determine if the mutations were successful at stopping the negative control protein from ligand switching upon reduction.

The variant WT*/N33/Q39/I52/C80 was lysed and centrifuged, obtaining an intital cell---lysate volume of 100.0mL. The protein was purified using ammonium sulfate precipitation, dialysis, and column chromatography. At this stage the protein was a pale orange/brown color. The protein was concentrated to a darker orange/brown color, and a UV---Vis spectrum was recorded to determine the protein yield, 6.77mg. The protein was divided into 0.5mL aliquots, frozen in liquid nitrogen, and stored at ---80°C.

In order to test the ligand switching ability of the protein, it was diluted using the MOPS pH 7.0 buffer and the citric acid buffers at pH 6.0 and pH 5.0, because the ligand switch was more likely to be stopped under acidic conditions. After adding sodium dithionite to each sample, it was found that all three samples continued to be reduced. A final sample was prepared at pH 4.0, but this sample was able to be reduced as well. The negative control protein still had the ability to ligand switch, meaning further manipulation of the protein structure was still necessary in order to obtain a properly functioning negative control.

Results

The overall purpose of this research was to use cytochrome *c* to create a workable model of Type II proteins in order to study the mechanisms by which they undergo a ligand switch. An important step towards this goal was achieved by mutating the amino acid attached to the heme of the protein into a cysteine, which is

one of the defining features of this specific type of protein. The fact that this protein was successfully mutated and created was a positive result in the overall progress of this research project. However, an unforeseen problem arose in that the negative control protein was able to ligand switch. The development of a negative control is crucial for being able to study the mechanism of the ligand switch; therefore, the majority of the project was devoted to looking for possible reasons as to why the ligand switch was still occurring in the negative controls. Various mutations have proven to be unsuccessful in eliminating the ligand switch ability of the negative control; however, each potential ligand that is eliminated brings the overall goal of a workable control closer. Overall, even though improvements have been made to the model, the answer to the question of the unknown ligand switch remains unknown at the conclusion of this project.

Discussion

Two other methods for getting rid of various ligands have promise in helping with the protein development. The first is acetylation, which adds a blocking acetyl functional group to the amino acid lysine, as well as possibly blocking the amino---terminus of the protein. A small scale acetylation was attempted, but the protein was still able to ligand switch. Still, it is possible that this is a technique that could be better developed and attempted in the future to see if the results change. The second method involves more PCR mutations of the protein in an attempt to get rid of the amino acids capable of acting as heme ligands. Two potential sites of mutation are the histidine at the 26^{th} position, which had been ignored because its presence

added stability to the structure of the protein, and the amino---terminus of the protein. Because the amino terminal group has a nitrogen atom similar to the histidine amino acid residues, there is a chance that the end of the protein is actually the cause of the unwanted ligand switch. Mutations that cause a cyclizing effect of the amino---terminus may prevent it from ligand switching.

Conclusion

The objective of this research project was to create a model protein that could be used to study the mechanism of the ligand switch in Type II thiolate---ligated heme proteins. Type II proteins are a diverse group and they have been implicated in gas exchange, metabolism, and regulation of the circadian rhythm. There are several that can be tied to various disease states, such as CBS, and learning how the ligand switch controls protein activation and function could be crucial to learning more about the protein as a whole. Because of the complexity of the proteins of interest, a smaller, simpler model was to be created using cytochrome *c*. A fundamental step was taken towards the creation of a valid model by mutating the amino acid attached to the heme from a methionine to a cysteine. However, a setback occurred when it was discovered that the negative control protein was able to ligand switch as well. The remainder of the research was done in order to try and determine what the negative control protein is using as a ligand. Although many of the variables that could be causing this switch have been eliminated, the nature of the ligand switch remains unknown at this time. Further research will be conducted in order to determine the nature of this ligand switch, and once the negative control

is identified it can be removed. Then other protein models with specific ligands can be created and tested under varying environmental conditions. The information that is gained from the simple protein model can then be applied to the ligand switch of the more complex Type II proteins, and this will lead to a greater understanding of how these thiolate---ligated heme proteins function.

Literature Cited

Cherney, Melisa M. and Bowler, Bruce E. "Protein Dynamics and Function: Making New Strides with an Old Warhorse, the Alkaline Conformation Transition of Cytochrome *c." Coordination Chemistry Reviews.* 2010, *255:* 664---677.

Igarashi, Jotaro, *et al*. "The Roles of Thiolate---Heme Proteins, Other Than the P450 Cytochromes, in the Regulation of Heme---Sensor Proteins." *Acta Chimica Slovenica.* 2008, *55*: 67---74.

Marvin, Katherine A., "Characterization of Redox--- and Gas---Responsive Heme---Thiolate Proteins." Doctoral Dissertation, University of Wisconsin---Madison. 2008: 1---36.

Omura, Tsuneo. "Heme---Thiolate Proteins." *Biochemical and Biophyiscal Research Communications.* 2005, *338*: 404---409.

Pazicni, Samuel. "Towards Understanding the Role of the Heme Cofactor in Cystathionine β---Synthase." Doctoral Dissertation, University of Wisconsin---Madison. 2006: 1---48.

Raphael, Adrienne L. and Gray, Harry B. "Semisynthesis of Axial---Ligand (Position 80) Mutants of Cytochrome *c*." *Journal of the American Chemical Society.* 1991, *113:* 1038---1040.

Shimizu, Toru. "Binding of Cysteine Thiolate to the Fe(III) Heme Complex is Critical for the Function of Heme Sensor Proteins." *Journal of Inorganic Biochemistry.* 2012, *108*: 171---177.

Figures

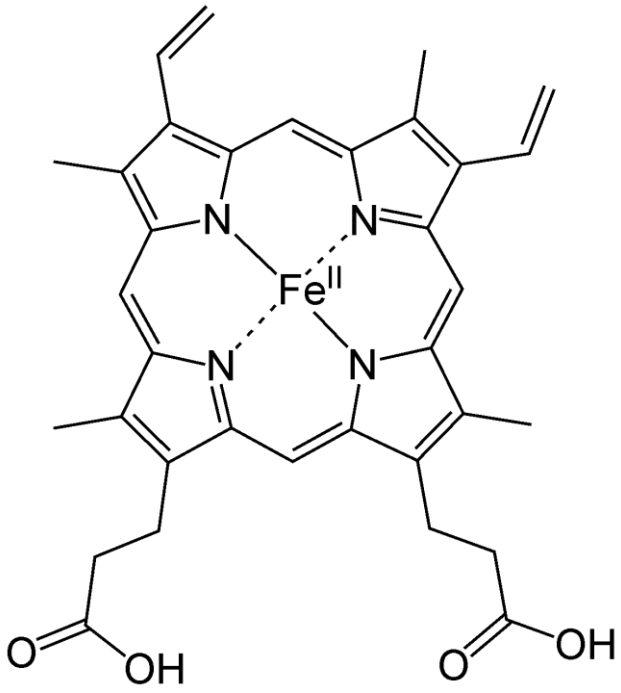

Figure 1: A model of heme; it is an iron bound by a porphyrin ring. The iron can form six bonds total in a Type II protein: four with the nitrogens in the ring, and two with the protein itself (above and below the plane of the image).

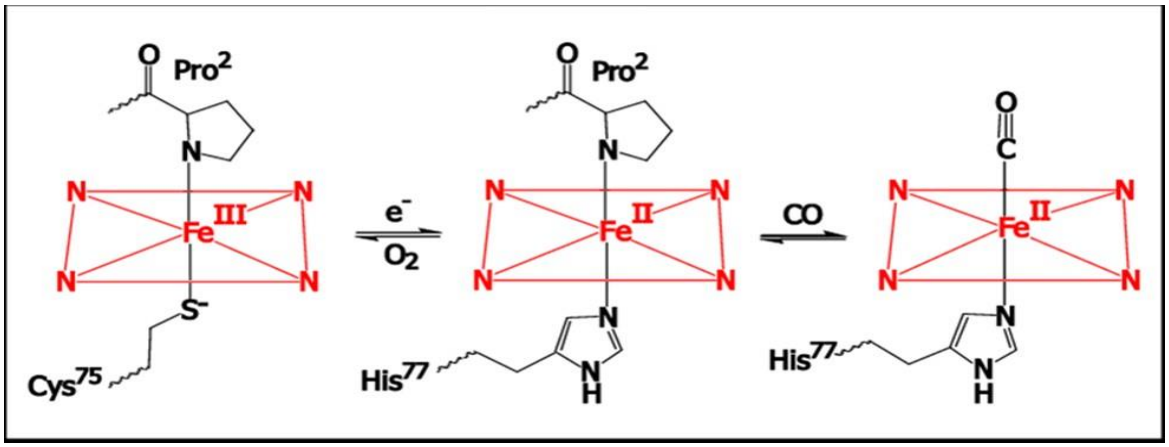

Figure 2: Ligand Switch Mechanism in a Type II Protein, CooA. This protein has a ligand switch that replaces the cysteine (Cys^{75}) with a histidine (His^{77}). This causes the proline (Pro^{2}) on the other side to dissociate, and that side of the iron is open to bind carbon monoxide, which activates the protein and allows the bacterium to use carbon monoxide for metabolic purposes.

Protein	Protein function	Heme function	Role
CooA	transcription factor	CO sensing	anaerobic growth
RcoM2	transcription factor	CO sensing	anaerobic growth
E75	transcription factor	redox/gas sensor?	development
DHR51	transcription factor	redox/gas sensor?	?
Rev-Erbα & β	transcription factor	heme sensor?	circadian rhythms
NPAS2	transcription factor	CO sensing	circadian rhythms
PER2	transcription factor	heme sensing?	circadian rhythms
Bach1	transcription factor	heme sensing	heme metabolism
IRP2	mRNA binding	heme/redox sensing	iron homeostasis
DGCR8	RNA binding	heme sensing?	pre-miRNA cleavage
HRI	kinase	heme/$NO^{\cdot}$ sensing?	protein synthesis
CBS	synthase	???	metabolism
Slo1 BK channel	ion channel	heme sensing?	K^{+} transport

Table 1: Several known Type II proteins with their functions.

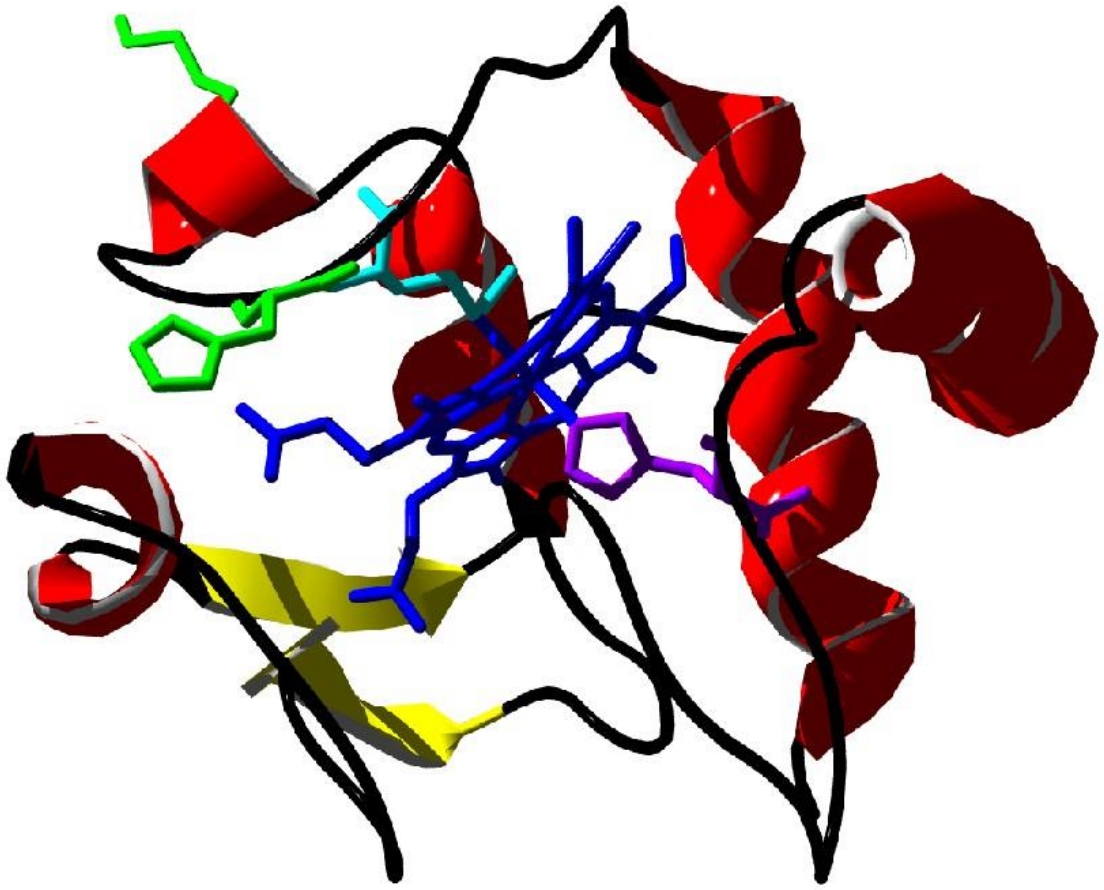

Figure 3: A three---dimensional model of cytochrome *c*. The heme is in blue, and several key amino acids are shown, including Cys80 (above the heme) and His73 (amino acid with the pentagonal structure).

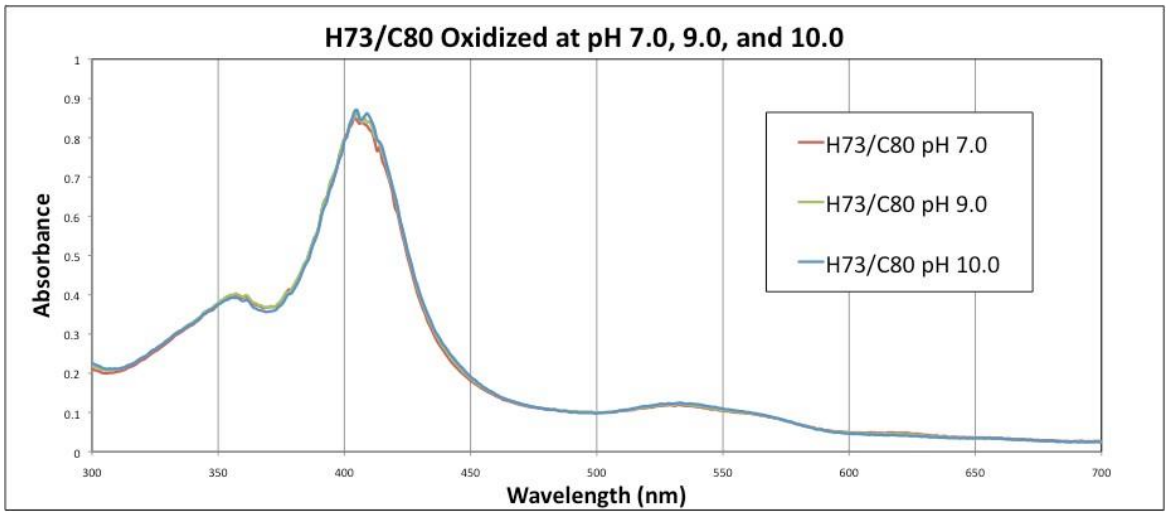

Figure 4: An example of a UV---Vis spectrum of WT*/H73/C80 at three different pH values. This shows that pH had little effect on the heme ligation. The distinct shape of the spectrum indicates that the heme is in an oxidized (Fe^{3+}) state.

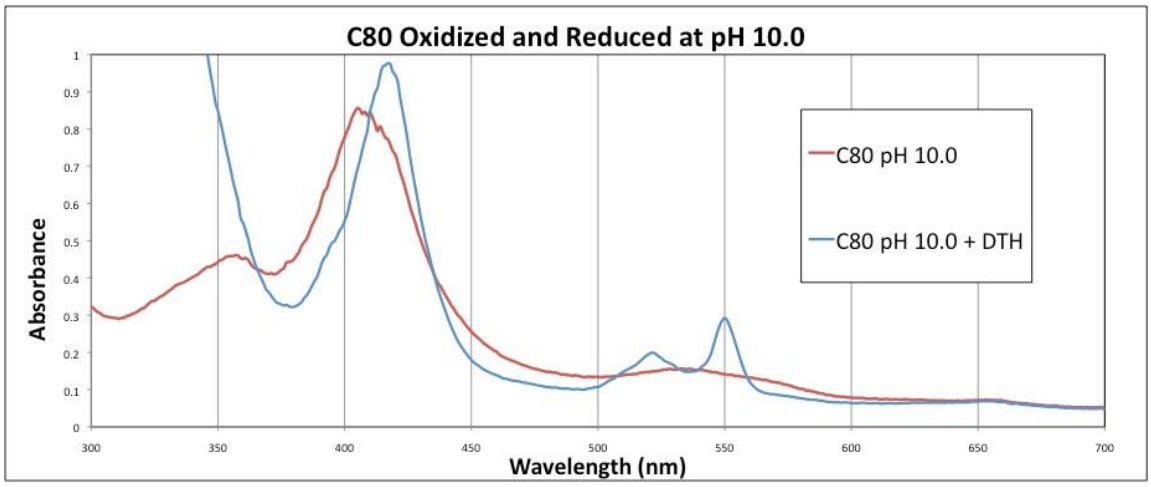

Figure 5: The spectrum of WT*/C80. This shows the clear differences between an oxidized (Fe^{3+}) and reduced (Fe^{2+}) state. This was unexpected for this variant, because it should not have been able to ligand switch, and therefore was unlikely to be reduced by the addition of sodium dithionite (DTH).