Continued Study of Proton Pumping by Incorporation of a Genetically Encoded Infrared Probe

UROP Assistantship Proposal Fall 2015

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Abstract

The mechanism by which a proton-pumping enzyme transports protons across a membrane is still unknown. The proton-pumping enzyme ubiquinol oxidase (UbO) will be studied using a new technique that combines molecular biology with spectroscopy. An unnatural amino acid, para-cyanophenylalanine (pCNF) will be incorporated genetically into the sequence of ubiquinol oxidase and the enzyme will be observed with various active site heme oxidation states by Fourier Transform Infrared Spectroscopy (FTIR). The information gained from these experiments will be used to analyze the conformational changes undergone during the proton-pumping process and to determine the mechanism of proton pumping.

Background

Proton-pumping is a vital bioenergetic process that is carried out by enzymes known as proton pumps. It is the mechanism by which protons are pumped from inside the mitochondrial matrix, against the electrochemical gradient, to the intramembranous space. This buildup of protons in the intramembranous space allows electrochemical energy to be converted to stored energy in the form of ATP as a proton flows back into the mitochondrial membrane through ATP synthase. The ability to store and transfer energy in cells is vital to maintain homeostasis, and ATP is the central molecule used for energy transfer and short-term storage in biological systems. Determining the mechanism by which protons are pumped across the mitochondrial membrane is important to understanding the generation and transformation of energy on the cellular level.

An example of a proton-pumping enzyme is cytochrome c oxidase (CcO). This enzyme is found in the mitochondrial membrane of eukaryotic cells and is an important enzyme in the electron transfer cascade, where oxygen (O_2) is reduced to water in the cellular respiration. Cytochrome c provides the electrons to drive this reduction, and electrons are transferred by

redox centers to the active site of the enzyme in concert with proton pumping[1], resulting in the pumping of four protons[2] across the mitochondrial membrane as shown below:

$$4\,Cyt.C.(reduced) + O_2 + 8H^+ + 4e^- \rightleftharpoons 4\,Cyt.C.(Oxidized) + 2H_2O + 4H^+$$

Another proton pumping enzyme is ubiquinol oxidase (UbO). It is an enzyme found bacteria such as *E. coli*. Ubiquinol oxidase is similar in structure to cytochrome c oxidase, though it uses quinol as an electron donor to reduce oxygen rather than cytochrome c[9]. It is easier to obtain and to isolate than CcO, so it will be the primary focus of this study. The mechanism by which an enzyme, such as CcO or UbO, transports protons across a membrane against the electrochemical gradient is still unknown [3], but it is postulated that a proton pump must have a channel to allow the flow of protons, a valve or gate site that prevents the flow of protons back through the channel, a pumping site, and an energy source [4,5].

A combination of genetic modification and spectroscopy will be used to determine what occurs within UbO as proton pumping is occurring. Genetic incorporation of the unnatural amino acid para-cyanophenylalanine (pCNF) in place of similar amino acid residues within a protein is a way to install infrared probes within the structure of a protein. This unnatural amino acid has a nitrile group ($C \equiv N$), which is an effective probe because its vibrations responds to changes in the electrostatic environment [6,7] such as changes in solvent [10] or distribution of protons, or motion of a prosthetic group (see Figure 1). It is also small and therefore unlikely to disrupt natural protein folding and function [8]. Its vibrations can be detected in the infrared region at around 2230 cm^{-1}, and the

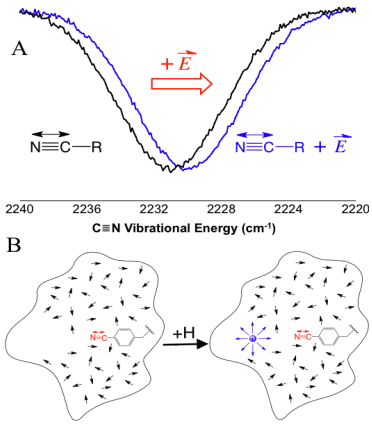

Figure 1. (A) Expected vibrational shift due to change in electrostatic environment.
(B) A pictorial representation of the change in electric field as a proton approaches the CN probe in pCNF

signal is unique from other signals associated with natural amino acid residues. This allows the detection and quantification of vibrational change as the electronic environment of a protein changes, or as the protein moves. Observing the shift in the $C \equiv N$ vibration could also potentially reveal key protonation sites on ubiquinol oxidase during proton pumping.

Eventually, time-resolved resonance Raman spectroscopy will be used to observe proton pumping. However, before that can be done, several well-defined oxidation states of the heme o_3 in UbO's subunit will be studied. The three states that will be studied are the reduced state (R), the oxidized state (O), and P_M state [11]. Ubiquinol oxidase will be purified and each state will be isolated and analyzed by FTIR. This experiment will provide important information about the effectiveness of the probes in observing the conformation of these states. This information will be vital in evaluating any vibrational shifts observed by Raman spectroscopy while proton pumping is occurring.

Two plasmids are used to incorporate pCNF into the amino acid sequence of ubiquinol oxidase, pUbO-pCNF and pSup-TRN. The former contains the amber codon in an appropriate location, and the latter plasmid codes for the appropriate acyl transferase to incorporate pCNF at the amber codon in pUbO-pCNF. Last semester, DNA from a strain of E. Coli containing the plasmid for incorporating pCNF in place of the Tyr173 (Y173F*) residue was purified. This DNA was transformed into competent cells (DH5α) and the mutant UbO was expressed and purified. Proton-pumping activity and quinol oxidation assays were performed to determine the functionality of the mutant enzyme, but FTIR spectra have not yet been obtained. Additionally, ubiquinol oxidase was purified from wild type cells to serve as an experimental control.

In addition to the plasmid Y173F*, plasmid stocks of several single point mutations have been prepared to replace the residues at the 112, 113, 420, and 335 locations. These plasmids

have not yet been expressed into proteins. Because the 112 site is near the proposed gate, incorporating pCNF at this site will be useful in experimentally verifying the location of the gate site. The 420 site is also an interesting site because it is situated between two hemes (b and o_3) which function as redox centers.

Specific Goals and Timeline

August-September 2015	The Y173F* mutant protein will be analyzed by Fourier Transform Infrared Spectroscopy (FTIR). Work will begin on cotransforming the stock plasmids F112F* and F420F* into the RG145 expression strain.
October 2015	Express and purify F112F* and F420F* ubiquinol oxidases. Purification will be accomplished using FPLC (Fast Protein Liquid Chromatography). The FPLC column used will be a nickel column, which has an affinity for the histidine tag that will be incorporated to the Ubo during expression.
November 2015	Verify the purification and identities of F420F* and F112F* ubiquinol oxidases by FTIR or mass spectrometry. Perform proton pumping and quinol oxidation assays. Begin expressing other mutants.
December 2015	For F112F* and F420F* UbO, the O, R and PM oxidation states will be isolated and analyzed by FTIR. Other mutant proteins will be purified.

Relationship to Faculty Mentor Expertise

Dr. _____ researches metalloproteins and their functions to better understand energy storage and transfer in biological systems, which knowledge could solve problems related to health and environmental problems or could inspire new modes of catalysis that could impact other areas of technology. In addition to studying mechanisms such as proton pumping, his research also includes studies of photosynthetic reactions to explore the possibility of artificial

photosynthetic catalysts, as well as ethylene sensing in plants, which has numerous applications in areas from biofuels to small molecule signaling.

My Goals

I am currently a senior majoring in Chemistry and Chemical Engineering. I have developed an interest in biochemistry and the applications of biochemistry and genetics in engineering disciplines, such as biofuels and renewable energy. I plan to attend graduate school and continue my studies in areas at the interface of biochemistry and engineering. Continued research and work with the UROP program will help me to achieve my goal to complete a graduate degree as well as help me to gain skills and experience that will be valuable in my profession. Though I do not know exactly what I will do in my career, I hope to be able to use the skills and knowledge I obtain to contribute to the well being of society and the environment.

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