FIG. 1.

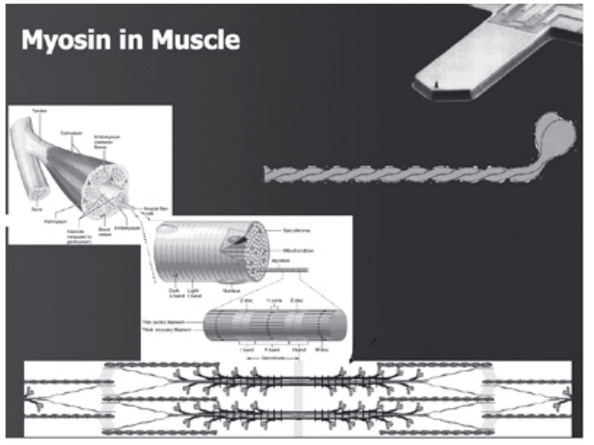


FIG. 2.

FIG. 3.
RESULTS: MICROSCOPY

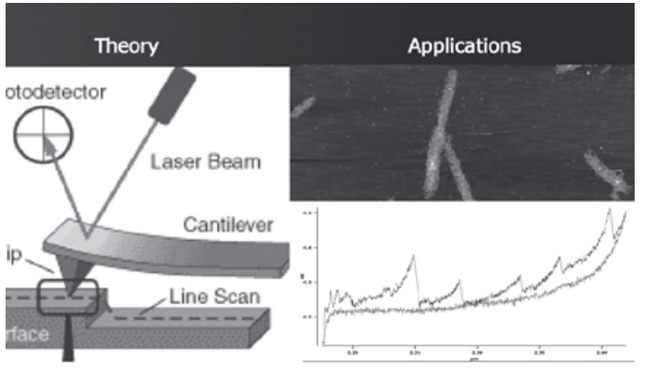
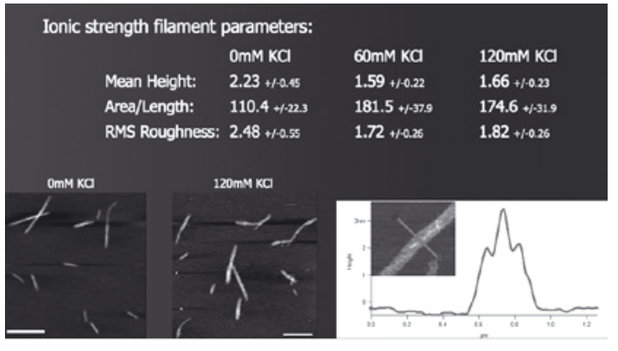


FIG.4.
RESULTS: MORPHOLOGY



Phosphoglycerate Kinase

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Biophysics is a discipline concerned with how cellular processes function. Two general questions that biophysicists seek to answer are: 1) How does a protein, with a given amino acid sequence, fold into its desired conformation. 2) In its native conformation, how is the protein folded, and how does it function. This paper discusses the techniques and experiment used in attempting to address certain aspects of these questions. The protein of focus for these experiments was phosphoglycerate kinase. Techniques used include, fluorescent labeling, electron paramagnetic spin labeling, and amyloid formation. In addition, comments and observations about performing research in Hungary are included.

1. Introduction

My interest in coming to Hungary on the Fulbright grant was in performing medically related research. I was generously given this opportunity in the Department of Biophysics and Radiation Biology at Semmelweis University. The goal of this paper is to explain the research I have performed, but to do so in a way that is available to all audiences. The reason for this is two-fold: First, making science accessible is important to me and an advantageous skill to posses; people care more if they can follow what is happening. Second, at this juncture, I do not believe I have enough accomplished

to present a purely scientific paper. Therefore, we will begin with an overview of proteins, and an introduction to the specific one studied. Then, we shall move to the experimental techniques, and how they are used to perform the research. Hopefully, this will be an interesting and informative read.

2. Protein Introduction

Proteins are a crucial part of life and have a role in almost all cellular processes. Many proteins fall into the class of enzymes. These proteins catalyze reactions by providing a favorable environment for them to take place. The enzyme may change structure or shape during the reaction but, upon completion, the enzyme returns to its original form. The protein studied falls into this class, and the words protein or enzyme may be used interchangeably in this paper.

The building blocks of proteins are amino acids. As the name suggests each amino acid contains an amine group and a carboxylic acid group. Each amino acid also has a side chain which gives each amino acid its distinct property. A protein is simply a long string of amino acids. The sequence of amino acids, for any given protein, is encoded in a cell's DNA. This sequence determines the structure of the protein by their interactions. The preferred structure or conformation, where a protein can perform its function, is referred to as folded, or native. Logically, a protein may not be in the correct conformation, and is thus called unfolded or non-native. It is still an open question in biophysics how a protein goes from unfolded to folded as quickly as they are observed to do. Along with the structure and function of proteins, this folding process is an important aspect of biophysics.

3. Phosphoglycerate Kinase

All of the research that has been conducted involves a single protein, phosphoglycerate kinase. Phosphoglycerate kinase (PGK) is a ubiquitous enzyme that is found in glycolysis, the breakdown of glucose, of most organisms. This process describes how usable energy, in the form of adenosine triphosphate (ATP), is gained from glucose. The step that PGK is responsible for catalyzing is the reversible transformation of 1,3 bisphosphoglycerate (1,3 BPG) to 3-phosphoglycerate (3PG). This conversion is carried out by transferring a phosphate group from 1,3 BPG to an accepting adenosine diphosphate (ADP) molecule. This is an example of substrate level phosphorylation, because the PGK takes the phosphate directly from a substrate, and attaches it to the ADP.[1]

Yeast PGK is composed of 415 amino acid residues, and is divided into two main domains each consisting of approximately 200 amino acids. The 1,3BPG binding site is located in the N-domain, the beginning of the sequence, and the ADP binding site is located in the C-domain, the end of the sequence. PGK functions as a hinge bending protein, where the protein flexes bring the two domains together to perform a phosphate transfer[2]

Aside from the intrinsic value of knowing how this enzyme functions there are several reasons for studying this protein. PGK has been shown to preferentially phosphorylate L-nucleosides, a class of medicines used as anti-cancer and anti-retroviral drugs.[3] Also, in erythrocytes, red blood cells, infected with malaria, PGK is highly depended upon by the bacteria to supply it with energy.[4] Gaining a better understanding of PGK is essential to understanding its roles in these functions.

The majority of the studies have been performed on yeast PGK. However, this protein is used as a model for human PGK because of its ease of use. Future studies on human PGK are planned.

4. Protein Expression

The first task in beginning to study PGK is to be able to express, or create, the protein. Plasmids, DNA that codes for PGK, for wild type and mutant strains were created earlier and available in the lab. The expression of PGK was done using Escherichia coli, competent cells. These are weakened E. coli cells that are created to be porous and allow plasmids to permeate their membrane. The initial step in expressing the PGK proteins is to prepare the cells for the plasmids to enter. The E. coli cells are placed with the plasmids on ice for around 30 minutes. The combination of plasmid and competent E. coli cells are then heated at 42 degrees centigrade for 45 seconds. This allows the plasmids to enter the cells and begin to be expressed. To allow the cells to recover from the cold and heat and begin expression, the cells and plasmids are allowed to shake at 37 degrees centigrade for one hour.

As stated before, the goal of this transformation is to get the E. coli cells to manufacture our desired protein. Thus, the only desirable cells are those that contain the inserted plasmid. To ensure that only these cells will be allowed to survive, the plasmid contains genetic information for PGK along with genes that confer resistance for specific antibiotics to the E. coli cells. In this expression the antibiotics used were Kanamycin and Chloramphenicol. These antibiotics are added to all materials used to grow the cells, and thus weed out the cells that do not contain the plasmid.

The first place where the cells are grown is on agar plates. These consist of an agar gel with food for the cells including salt, peptone, and yeast extract. The agar, makes the solution gel, after boiling and cooling. After making these gel plates in petri dishes, the E. coli that was shaking is plated onto the agar gel so that cultures, colonies of bacteria, can begin to form. These plates containing the cells are incubated at 37 degrees centigrade until cultures form. Typical times are 6-7 hours or overnight. It is important not to let the cultures overgrow because each colony derives from a single cell and are thus genetically identical.

After cultures form on the agar plates, starter colonies are begun. A starter colony allows the cells to replicate so that more protein can be produced. The started colonies consist of approximately 15 ml of

solution made again from peptone, yeast extract and salt. As before, antibiotics are added to the solution to eliminate E. coli cells that did not take up the plasmid. To this solution containing antibiotics and broth, a single cell culture is added by removing it from the plate with a sterile pipette tip. Usually, two starter colonies are begun from the same cell culture. These starter cultures are allowed to grow overnight. They are shaken at 37 degrees centigrade during this period of growth.

After the starter colonies have grown they are transferred into a larger amount of broth. This broth contains the same ingredients as the starter colonies. Typically, around three liters of broth are used to express the final amount of protein. Once the starter colonies are transferred to this larger amount of broth they are allowed to shake for 3-4 hours at 37 degrees centigrade, again increasing the cell concentration. After this period Isopropyl βD-1-thiogalactopyranoside (IPTG) is added which induces the E. coli cells to produce protein. After the addition of IPTG the mixture is allowed to shake for an additional 4 hours. At this point the cells have multiplied significantly and contain the desired PGK.

The next step in the process of obtaining the PGK is to harvest the cells. This is done manually with the assistance of a centrifuge. Equal portions of the mixture, containing broth and cells, are loaded into centrifuge tubes and centrifuged for 15 minutes at 4000g. The dense cells, containing the protein, form a pellet at the bottom of the tubes while the supernatant, liquid portion, can be discarded. After all the liquid portions have been removed the cells are harvested and stored at -80 degrees centigrade in clear plastic tubes. The wet mass of the cells is calculated, and is typically between 15-20g.

5. Protein Purification

After the proteins are expressed and contained in the E. coli cells the next task is to separate the desired protein, in this case PGK, from the rest of the cellular components. This is done through a process known as column chromatography. Chromatography can be used to separate substances based on a variety of qualities, such as size, charge or affinity. To aid in separation of the sample, a His-tag was fused to the yeast PGK protein. This is a sequence of amino acids, all histidines, which occur at the end of the protein sequence and aid in purification. This tag was added when the plasmid coding for PGK was created. The column used for this purification is packed with a gel containing nickel ions. The amino acid histidine binds very well to these nickel residues. Thus, by having the PGK protein with six extra histidines it binds more readily to the column packing material than anything else and can be separated from the rest of the cellular components.

The first step in this chromatography is cracking the cells to release all the proteins inside. This is done with a sonicator that uses ultrasound to break the cells' membranes. This works wonderfully, but it also releases enzymes inside the cell

whose job it is to break down proteins. These enzymes are called proteases. To prevent these proteases from breaking down the PGK, protease inhibitors are added before the sonication takes place. After sonication the proteins and all the cellular components are basically in a thick soup. To remove the larger portions, the mixture is centrifuged. The protein is soluble in the buffer used for sonication so it remains in the supernatant after centrifugation. Centrifugation is performed several times, each time keeping the supernatant and discarding the pellet. Once all the insoluble pieces are removed from the mixture it is ready to be separated on the column.

The column is connected to and controlled by a FPLC (Fast Protein Layer Chromatography) machine. For the purification of yeast PGK the His-tag was used to separate it from the rest of the mixture. However, once the protein is bound to the column beads it needs to be removed, or eluted. The compound imidazole interrupts the interactions between the histidines and the nickels ions by binding strongly to the nickel itself. To begin the purification, the column is equilibrated with the loading buffer consisting of sodium phosphate and salt solution. Then the solution containing the protein is loaded onto the column. Samples and buffers are loaded onto the column by a pump that allows the flow rate of the solutions to be specified. Once all of the protein solution has been loaded on the column a second solution containing sodium phosphate and salt but also imidazole is then pumped through the column. The imidazole concentration is increased stepwise by combining the solution that contains imidazole with the solution lacking imidazole at differing concentrations. The reason for the stepwise addition of the imidazole is to release other proteins and cellular components that have adhered to the column. Almost all proteins contain histidines, so most will adhere to the column. However, since the number of histidines increases the strength with which the protein adheres, regularly occurring proteins, which at most will have two maybe three histidines in a row, are eluted at lower concentrations of imidazole than the His-tagged PGK. An added feature of the FPLC is that the solution flowing out of the column is monitored with ultraviolet (UV) light. The benefit of this is that the amino acid residue tryptophan absorbs UV light. Therefore, it detects when protein is being eluted from the column. All of the solutions being pumped through the column are sent through a series of monitors that assist in determining the contents of the eluent. After all the sensors are passed, the eluent is collected in test tubes of specified volumes around 10-15 ml. These collected volumes of solution are known as fractions. If everything has functioned correctly, the protein is contained in one or more of these fractions with little contamination.

6. Locating the PGK

Determining which fractions contain PGK can be difficult. The first tool used

to locate the protein is the UV absorbance meter of the FPLC. If a fraction is shown to have high absorbance then it is highly likely that this fraction contains protein. Also, since the PGK contains a His-tag the desired protein will likely occur later in the purification where the imidazole concentration was higher. After deciding which fractions should be checked an electrophoresis gel is run on the different fractions. Electrophoresis consists of a polyacrylamide gel, with lanes where samples can be loaded. The gel operates as a filter, allowing small particles to move easily through, while slowing down larger molecules, and thus separating molecules by size. Small samples of each fraction, along with a staining solution, are loaded into these lanes then the gel is placed in a uniform electrical field. This electrical field causes the proteins, which are charged, to flow through the gel, and separate according to size. By comparing where bands show up for each fraction to a ladder with known piece sizes, the fractions containing PGK can be determined. The fractions with the most PGK and least contaminants are selected and used for protein samples.[5]

7. Dilution and Concentration Determination

After the fractions are chosen they are ultra-filtrated to increase the protein concentration in solution. This is easily done with a sensitive filter that allows buffer to pass through but restricts the protein. The fractions are reduced to a volume of several milliliters.

The next task is to determine what concentration of protein is in the solution. This is done by measuring the absorption of the sample at a wavelength of 280 nm. The absorption value is compared with a standardized absorption constant where 1g of PGK per 100ml and with a cuvette pathlength of 1 cm has an absorption value of 4.95.[6] This comparison gives the concentration of PGK in the sample. This information is useful in determining the activity of the protein, and in knowing the amount of label that needs to be added in future experiments.

8. Activation Measurements

One of the major tests to tell if the PGK is a functioning protein is to perform activity measurements. As stated before, PGK is responsible for the step in glycolysis where 1,3BPG is transformed into 3PG. This reaction requires ADP to take on a phosphate and become ATP. However, often glycolysis is reversed, albeit with some different enzymes. This process is gluconeogenesis, the production of glucose from its components[7]. It is this reverse reaction that is used to test the PGK's activity, and it involves a series of reactions. The first step in the activity measurement is when PGK removes a phosphate group from ATP and adds it to 3PG to form 1,3BPG. The 1,3BPG is then sent on to glyceraldehyde 3-phosphate dehydrogenase (GAPD), for the next step in the reaction. Similar to the last reaction, a phosphate is removed to form glyceraldehyde 3-phosphate (G3P). However, this reverse reaction requires a reducing agent to occur. This reducing agent is nicotinamide adenine dinucleotide (NADH), and it donates its hydrogen to the help the reaction occur. Upon donation of this hydrogen NADH becomes NAD+, and this is the key to monitoring the activity of PGK. NADH absorbs light at a wavelength of 340 nm but NAD+ does not. Thus, if the absorption of the reaction at 340 nm is monitored, the rate at which NADH is being consumed can be determined. Since one NADH molecule is consumed for every 1,3BPG that is transformed, and 1,3BPG only comes from PGK activity, each NADH consumed by the reaction implies that the PGK has performed its function at least once. So to measure the activity of the PGK we combine excess 3PG, NADH and GAPD, into a cuvette and monitor the absorption of 340 nm light. This technique has been used to test whether the proteins that have been expressed, or had experiments performed on them, are viable. A protein, normally, can only function at full capacity if it is in the correct conformation. So the activity measurement yields information on the state of the protein.

10. ATP-labeled Dye

The source of energy within biological systems is largely contained in the molecule ATP. The chemical potential within this molecule can be used to carry out a variety of cellular functions from muscle contraction and movement of macromolecules, to synthesis of DNA. The amount of ATP consumed can approach a person's bodyweight per day, and it is continually being replenished.[8] It is of interest how this chemical energy is accessed to produce these changes. Often times, the chemical energy is used by enzymes to perform their function, through small changes in the binding pocket of the enzyme. PGK can be used as a familiar model system to study this interaction. An attempt was made to examine the function of PGK and its interactions with ATP.

As stated earlier during gluconeogenesis PGK takes 3PG and ATP and forms 1,3BPG. To perform this experiment a florescent ATP analog, Cy3-EDA-ATP, was used.[9] The ATP analog absorbs and emits different wavelengths of light, depending on how the analog is embedded in the protein. This happens because the analog's environment determines what energy state the molecule is in, and thus the wavelength of the light emitted. The light emitted is collected in a spectra, with peaks occurring where the most light is emitted. The goal is to follow these peaks, and watch how they change as the dye is excited at different wavelengths of light.

At standard temperatures PGK is continually opening and closing, attempting to carry out the phosphate transfer. Thus, to be able to get distinguishable peaks the protein must be frozen in specific conformations. To achieve this, the samples are cooled to cryogenic temperatures, around

10 K. This freezes the PGK in its current conformation, and allows static conformations to be examined. In addition to freezing the protein, the motion of the protein's environment, which causes fluctuations, is also eliminated.

It is hypothesized that PGK occurs in two conformations, open and closed. The open conformation is expected to predominate when one or none of its substrates are present. Conversely, the closed conformation should be preferred when both substrates are present. These two conformations, and possibly others, should present themselves as distinct peaks, with differing emission intensities, on the collected emission spectra. The intensities of the peaks should indicate how the conformation preference of PGK changes, depending on the substrates available.

Experiments were conducted under several conditions. The experiments were done in two different types of buffer, tris buffer and phosphate buffer. Experiments were also performed on just the ATP dye, ATP dye and PGK, and ATP dye, PGK and 3PG. In each experiment glycerol was used to discourage scattering. In a purely aqueous environment, crystals would form and disrupt measurements. Making half the volume glycerol, which will not form crystals, limits this inhibition.

Unfortunately, these experiments did not yield as much information as initially hoped. The instrument, the Fluorolog, used to excite the ATP analog and collect the florescence was not precise enough to distinguish between some peaks. For example, two sharp peaks could show up as one broad peak. Information that was useful, however is the range over which these peaks occur. The next step is to use a laser, which has a greater resolution, to examine specific peaks, but over a smaller range.

11. Amyloids

One major area of biophysics is determining the pathway by which proteins fold and unfold. Often times, proteins become stuck in different conformations and are thus non-viable, and can often cause disease. Proteins may become unfolded and form aggregates because of interactions between their amino acids, under certain conditions. Proteins contain two types of amino acids: Ones that are hydrophilic, meaning they prefer the polar environment of water, or hydrophobic, meaning they prefer to avoid the polar environment of water. The environment of most proteins inside physiological cells is aqueous. Thus, the hydrophilic amino acids prefer to be near this environment, while the hydrophobic ones shun it. A protein's native conformation accommodates the amino acids by burying the hydrophobic amino acids inside of the folded protein, and allowing the hydrophilic amino acids to be exposed to the aqueous environment. This attraction between hydrophobic amino acids can cause problems when proteins become unfolded. When a protein is unfolded, usually occurring under harsh conditions, it is not in its native conformation. Thus, hydrophobic amino acids will be exposed to unfavorable environments. This causes the hydrophobic amino acids to be attracted to each other and form protein aggregates. If these protein aggregates have certain properties they are termed amyloids. Amyloids proteins have the unfortunate property of being responsible for several diseases including Alzheimer's disease, Parkinson's disease, and many others.[10]

While yeast PGK is not involved in known disease pathways it can be used as a model to create and detect amyloids. To begin, yeast PGK is expressed and purified using the method described earlier. After the PGK is purified the protein is subjected to activity measurements. This ensures that functioning PGK is purified and that amyloids are formed from native protein. After this initial measurement is taken the proteins are then unfolded and formed into amyloids.

The creation of amyloids is accomplished by manipulating their environment. Protein structure is dependant on the formation of hydrogen bonds between different amino acid residues. By placing the proteins in a proton rich, low pH, environment it disrupts these hydrogen bonds and allows the protein to unfold. The conditions used to form amyloids in this experiment were pH 2.0, and salt concentration of 0.2 M (moles/liter). Adding the salt increases the ionic strength of the solution. When proteins are highly protonated they carry a positive charge and repel one another. Greater ionic strength allows the proteins over come this repulsion, combine and form amyloids. Exposing the proteins to this environment was accomplished through dialysis. The protein, which is dissolved in solution, is put into a membrane permeable by the buffer. This allows the concentration of protein to be preserved while subjecting it to the desired conditions.

To ensure that amyloids are indeed formed, the proteins were examined under an electron microscope. On a small copper grid, which was equipped with a carbon coated membrane to hold the proteins, a 100 fold dilution of the protein was loaded. After allowing the sample to settle on the membrane for 15 minutes the solution was removed. Then uranyl acetate was added as a negative stain. This means that the uranyl acetate will embed on the membrane, giving a contrast between sample and the background. The stain was also allowed to settle onto the membrane for 15 minutes and then removed. The grid was then placed in the electron microscope. In an electron microscope a beam of electrons is shot at the sample. The electrons are absorbed readily by the uranyl acetate but pass through the protein. This results in lighter areas where the protein is located and darker areas for the stain. When this procedure was carried out for the amyloid proteins their presence was confirmed by fibrous light areas from the electron microscope. As a control, protein that was not subject to the amyloid-forming conditions was tested, and showed no such fibrous areas.

Now that formation of amyloids was confirmed, further studies are planned with the PGK amyloids.

12. Electron Paramagnetic Resonance Labeling

Another current project underway deals again with labeling PGK. This time however, it is with an electron paramagnetic resonance (EPR) or electron spin resonance (ESR) label. This is a label that is attached directly to the protein and is sensitive to changing magnetic fields. The label contains an oxygen molecule with an unpaired electron. This electron is sensitive to changes in a magnetic field and, possibly, will yield information about the protein. The information that EPR yields is about how quickly portions of the protein move. This could lead to insights on the function of PGK.

The label being used for the experiment is 4-maleimido-TEMPO.[11] The maleimide group reacts, highly specifically, with the amino acid cysteine, because it uniquely contains a thiol group. A thiol group is characterized by the presence of a sulfur atom. In yeast PGK only one naturally occurring cysteine is present. It is buried within the interior of the protein so, for the TEMPO label to reach it, the protein must be unfolded. The reactions possible pH dependence dictates that the unfolding of the protein is performed with the salt GuHCl. Combining the protein, TEMPO dye and GuHCl has been shown to successfully label the protein. The project hinges upon the fact of refolding the protein so that it achieves native activity after the label is attached. Thus far, PGK has shown markedly decreased activity after labeling occurs. Performing EPR experiments on misfolded PGK will not be as useful, so the current goal is to find the correct conditions, if they exist, in which to label the protein. As this project is only weeks old, at the time of writing, this obstacle has yet to be overcome.

13. Difficulties Encountered

There have been some impediments to conducting research during my time in Budapest. I do not offer these as a complaint, but simply something that has had to be worked around.

The first obstacle has been change; my project has shifted focus several times. The first time was upon arrival. I had agreed on a project with my advisor before arrival, but was informed that this line of research was no longer being pursued. Research on PGK was agreeable, however, and readily accepted. The first task, though, was to reassemble and calibrate most of the equipment. The department moved during the previous summer and was in a state of transition. It was interesting to be able to assist in this process, but research was delayed during this period. At this point, work with the fluorescently labeled ATP began. After it was determined that the laser would be needed, that project was halted. The laser's optical equipment has yet to be assembled. This is not the fault of the department, but an indicator of a larger difficulty. The parts have been ordered but have not arrived.

A significant amount of time was lost during this grant period due to the inability of the department to order materials. For some reason, one that I do not fully comprehend, the funds that the department had to work with were, in effect, frozen. There was much difficulty in paying orders for shipment, and the companies making the supplies often, understandably, had problems delivering without payment. The columns needed for the purification of proteins were delayed. The IPTG, used for expression of proteins, could not be order. The optical equipment for the laser still has not arrived. This has been the largest obstacle to overcome. I have done my best to use my time prudently, but at times, things have seemed out of my control.

14. Other Activities

Fortunately, not all of my time in Budapest has been spent in the laboratory. I have done a variety of things that may be of interest.

One of my first goals was to familiarize myself with the Hungarian language. Unfortunately this has proved more difficult than I planned, mostly because I have not devoted as much time as I would have liked. I, hopefully, have improved however. Many people have allowed me to practice with them, and I have used what little I know to buy train tickets, order meals, give directions, and even call and make reservations. In the few months I have remaining I hope to devote even more of my time to studying the language.

Another highlight of my time here has been attending the Introduction to Public Health course taught by fellow Fulbrighter, Prof. Kristie Foley. She kindly allowed me to audit her lectures and borrow the textbook. This has been especially interesting because next year I will be entering an MD/PhD program at the University of Iowa. I am considering a degree in the area of public health and this course gave me more to consider. I have almost completed reading the textbook she lent me, and am grateful for her help.

A final portion of my time here has been spent attending the Nazareti Egyház, the Hungarian Church of the Nazarene in Budapest. The church in Budapest doubles as the office for the entire Hungarian District. The church's pastor and his family have become my closest friends in Budapest and I am appreciative they accepted me into their church. They have allowed me to teach adult Sunday Schools classes, lead small group meetings, and serve as a representative for the youth of the Hungarian district. These activities have provided a nice balance to the research portion of my grant.

15. Conclusions

This grant period has been much different than I had originally expected. The difficulties and delays experienced were often frustrating and disheartening. However, the goal of the Fulbright organization is to promote mutual understanding between people of other countries and those of the United States. Hopefully, I have accomplished that during my time in Hungary. Also, I hope

that I have contributed to the Department of Biophysics in their ongoing research. I trust that the projects I have participates in will be continued after I leave. I have contributed to the instillation of the FPLC, which greatly reduces the time needed for protein purification. Also, in the coming weeks I will purify human PGK for work on a new enzyme.
However, I believe my biggest contribution may have been simply helping wherever I could. I have enjoyed working with my colleagues on their projects and am thankful they allowed me to do so. I have thoroughly enjoyed my time here in Hungary, and am thankful for the many people who made it possible.

16. Acknowledgements

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Analyzing Social and Economic Mobility for the Roma in Hungary: A Look at Government Initiatives and International Responses

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While many groups in Hungary have benefited from economic modernization and development, others, such as the Roma, have not benefited as much from these advances. The Roma are the largest minority in Europe and in post-Communist countries, such as Hungary, are known as the biggest 'losers' of the change from Communism to capitalism because of the loss of governmental welfare programs. Currently, in Hungary the Roma face obstacles and challenges in access to healthcare, education, employment, and adequate housing. However, over the past decade the European Union and the Hungarian government have taken steps to promote anti-discrimination legislation and social programs focusing on equality and diversity. Additionally, international non-governmental organizations have also played an important role in minority rights in Central and Eastern Europe providing advocacy, resources, and policy development.