Running head: CYANOBACTERIA

Protein Phosphorylation and a Novel Phosphatase in the Cyanobacterium *Anabaena*

Robert Mullis

A Senior Thesis submitted in partial fulfillment
of the requirements for graduation
in the Honors Program
Liberty University
Spring 2008

Acceptance of Senior Honors Thesis

This Senior Honors Thesis is accepted in partial
fulfillment of the requirements for graduation from the
Honors Program of Liberty University.

_____

L. Daniel Howell, Ph.D.
Chairman of Thesis

_____

Mark Hemric, Ph.D.
Committee Member

_____

Emily Heady, Ph.D.
Committee Member

_____

Brenda Ayres, Ph.D.
Honors Assistant Director

_____

Date

Abstract

The focus of this paper is a dual-specific protein phosphatase (DSP) previously found in the periplasm of the cyanobacterium *Anabaena* PCC 7120 that may regulate circadian rhythms in that organism. To fully understand the topic, summaries of enzyme action, cyanobacteria, circadian rhythms, and phosphorylation cycles are required and therefore discussed in this report before the presentation of previous and current laboratory research centered on the phosphatase.

A continuous cyanobacterial culture was maintained while cells were collected for harvesting periplasm. Tests to determine size, enzyme activity, and protein content were performed on the periplasm of the bacterial cells. Initial findings revealed at least one periplasmic protein phosphatase and possible optimal pH levels for each.

Table of Contents

Protein Phosphorylation and a Novel Phosphatase in the Cyanobacterium *Anabaena*

Proteins are truly marvelous molecules, making up a vast portion of the basic cellular composition. Life cannot exist without them, and every major function in every organism involves at least one protein. Proteins are the direct products of the genetic code and a prominent source of nitrogen in cells. Their importance is easily seen when one realizes that proteins are the victims of mutations. Deleterious mutations, or those that cause the death of the organism, sometimes affect only one of these molecules; therefore, their importance can never be downplayed.

This paper focuses on a protein phosphatase discovered by researchers studying tyrosine phosphorylation in cyanobacterial species of the genus *Nostoc* and the genus *Anabaena*. Initial work dealt with purifying and sizing the protein and preliminary dephosphorylation tests, which resulted in the discovery that the protein was dual-specific in its substrate preference. Experimentation also showed a possible circadian rhythm pattern of enzymatic activity by the phosphatase. These results, however, were left unpublished. This paper represents the first experiments aimed at confirming the presence and properties of the periplasmic phosphatase found in *Anabaena* PCC 7120, and it includes background information on enzymes, phosphorylation, circadian rhythms, and cyanobacteria, as well as the results of the initial experimentation performed in 1997. Because each piece of data which resulted from these experiments relates to the fact that this protein is an enzyme, that class of proteins will be discussed first, followed by information about the organism in question, *Anabaena*.

Enzymes

Before a pointed discussion about the dual-specific phosphatase can be made, a more basic definition needs to be given. Phosphatases are enzymes, which are proteins that carry out almost every major catalytic function in both eukaryotic and prokaryotic cells. These wonders of creation, when folded into a final, specific conformation after synthesis, possess an active site which allows the molecules to catalyze reactions. A single mutation in the gene leading to a single change in the protein sequence at or near the active site, or even at a distant site which interacts with the active site, can cripple the protein to the point that it no longer functions.

Enzymes accomplish their functions in the cell through catalysis. A cell performs thousands of different reactions which could theoretically occur without enzymes. The reaction rates without enzymes, however, would be insufficient to sustain life. Enzymes speed up reactions by holding reactants in place and distorting them into transition states. In doing so, the catalyst lowers the amount of energy required to form products from the reactants. In the theoretical situation above, the reactants must randomly meet in the cytoplasm and remain in contact long enough to react. As a case in point, urea will spontaneously hydrolyze to ammonia and bicarbonate at the rate of 0.0000000003 molecules every second, or slightly less than one molecule every century. If contact between reactants were the only consideration, the reaction would be spontaneous, because the cellular concentration of water is 55M. However, normal cellular energy is not high enough to twist the urea molecule into the transitional form. Urea would thus accumulate in the body without the enzyme urease, which raises the above rate to 30,000

molecules per second, or $1x10^{14}$ molecules per century. Therefore, in the presence of the enzyme, excess urea in the body quickly breaks down (Garrett and Grisham, 2005).

A second specializing property of enzymes is specificity. Havoc would reign in the cell if enzymes catalyzed reactions with every possible chemical and organic reactant in the cell. Each enzyme is therefore specifically designed to form complexes with only a small number of substrates, usually one or two, although some reactions utilize three substrates. In fact, the enzyme's specificity must be refined to the point that even the product formed cannot stably bind to the catalyst. Without this feature, products would rarely be released from the enzyme complex. Specificity stems from the exact shape of the active site on the protein, and this fact explains why mutations are so devastating to the enzyme's function. The site is both small in comparison to the size of the protein and three-dimensional in shape, and each site is specially designed to hold and interact with a small set of substrates (Copeland, 2000). In addition, no unwanted by-products are formed in a reaction catalyzed by an enzyme. This property sets enzymatic reactions apart from non-enzymatic ones, and it stems from the enzyme's specificity.

A final defining property of enzymatic systems in the cell is the level of regulation over those systems. The cell regulates enzymes by covalent and noncovalent means. The former type of regulation includes such activities as substrate or product concentration changes, enzyme concentration changes, end-product inhibition, repressing/activating subunits, and allosteric regulation. Concentration changes affect enzymatic rates by unbalancing the equilibrium between reactants and products; more product means a lower reaction rate, while more reactant means a higher reaction rate. A cell can also switch genes encoding enzymes on and off when needed to modulate

momentary cellular needs. End-product, or “feedback,” inhibition is a common form of regulation for long enzymatic pathways such as glycolysis. In these pathways, it is a general rule that the final product in the chain of reactions inhibits the enzyme which catalyzes a reaction earlier in the chain. Without this type of regulation, enzymatic pathways would process reactants nearly unchecked. Allosteric regulation entails a ligand-induced change in the quaternary structure of a protein in a way that makes further binding of substances more or less favorable (Matthews et al., 1997). The binding of oxygen to hemoglobin, though not an enzyme itself, is a classic example of allosteric modification.

Covalent modifications involve changes to the atomic composition of the enzyme itself. Zymogen activation in the cell represents an irreversible covalent modification. These “pre-proteins” are inactive until the cell needs an active enzyme, at which point the zymogen is proteolytically cleaved into the active form (Matthews et al., 1997). Reversible covalent modifications include phosphorylation, acetylation, methylation, and many other side-group additions. Phosphorylation, the subject of this paper, involves the addition of a phosphate group to the protein. This process will be discussed in further detail in the next section. Acetylation is the addition of an acetyl group, and methylation is the addition of a methyl group. Some additions, though included in the reversible covalent modifications category, cannot be removed under certain circumstances, making the changes effectively irreversible. This can happen when a modification to a protein results in refolding of the macromolecular structure that blocks access to the modified site (Matthews et al., 1997). The next section describes the specific enzymatic reactions of phosphorylation and dephosphorylation.

Phosphorylation

Phosphorylation is the process of transferring a phosphate group to a molecule which possesses a hydroxyl group, or a terminating –OH. Hydroxyl groups are present on three amino acid residues, but sugars are also regularly phosphorylated because of the multiple hydroxyl groups which characterize a sugar. A classic example of sugar phosphorylation is found in glycolysis, in which a glucose molecule must be phosphorylated twice before it is broken down to yield energy required by the cell. Kinases use the chemical energy from ATP (adenosine triphosphate) to activate the sugar with the phosphate groups, which is normally not spontaneous (Garrett and Grisham, 2005).

This study deals with the phosphorylation of serine, threonine, or tyrosine residues of a protein. Protein phosphorylation has many functions over a wide range of organ systems. Smooth muscle contraction, for example, utilizes protein phosphorylation. In this system of adding and removing phosphate groups, a kinase activates the light chain of the muscle protein myosin, which then contracts the muscle cell. Soon afterwards, a phosphatase deactivates the myosin light chain by dephosphorylation, and the muscle relaxes (Kitazawa et al., 1991). This process repeats itself countless times in the digestive and circulatory systems every day.

The major role of phosphorylation in the cell, however, is in signaling pathways, such as the activation of the protein p53 when DNA is damaged. In this case, phosphorylation leads to a cascade of further phosphorylation and acetylation of p53, which is followed by the regulation of cell cycle genes by the activated p53. Without this mechanism in place, the cell attempts to divide with damaged DNA and usually dies

when the broken DNA fragments upon division (Sakaguchi et al., 1998). A second pathway affected by phosphorylation is the activation of platelets when a clot is needed. Here, a protein tyrosine kinase $pp125^{FAK}$ is itself autophosphorylated before proceeding to phosphorylate the next protein in the cascade (Lipfert et al., 1992). Yet another pathway exhibiting phosphorylation is the T-cell activation in the immune response, where tyrosine kinases are actually regulated by tyrosine phosphatases in a cycle of phosphorylation and dephosphorylation (Garcia-Morales et al., 1990). Still another use for phosphorylation is the activation of caspase-9 in the apoptosis, or programmed cell death, pathway (Cardone et al., 1998).

The phosphorylation half of the cycle is catalyzed by proteins called kinases. Three major examples are protein kinase A (PKA), protein kinase B (PKB), and protein kinase C (PKC). The first, PKA, is intimately linked to cellular levels of cyclic-AMP and is made up of both regulatory and catalytic subunits. PKA has been linked to numerous functions in the metabolism of biomolecules such as lipids and sugars, and the cAMP-PKA pathway has been positively linked to human development (Reddy, 2005). PKB is implicated in specific functions such as insulin signal transduction and apoptosis prevention. In a more general explanation, PKB mediates both protein-lipid and protein-protein interactions (Alessi and Cohen, 1998). PKC has multiple cellular functions ranging from regulation of transcription, the immune response, and membrane action to receptor desensitization and the regulation of cell growth (Newton, 1995). Another specific example of kinase action is the phosphorylation activities of both hexokinase and phosphofructokinase during glycolysis. These two enzymes catalyze the “energy-input”

stages of glycolysis moving high-energy phosphate groups from ATP to the metabolizing glucose molecule (Garrett and Grisham, 2005).

Protein phosphatases catalyze the dephosphorylation half of the cycle. These proteins function to remove phosphate groups from serine, threonine, or tyrosine residues. Two major phosphatase families are PP1, protein phosphatase type 1, and PP2, or protein phosphatase type 2. These two subsets are found in organisms ranging from mammals to yeast and from flies to higher plants, and they help to prevent tumors by reversing effects caused by rampant phosphorylation by unregulated protein kinase C, although this is only one of the numerous functions of protein phosphatases in the body. Deleterious mutations of these proteins normally lead to the cessation of mitosis in the cell (Cohen and Cohen, 1989). PP1 phosphatases also play a role in processes ranging from glycogen metabolism to protein synthesis to intracellular transport. PP2 enzymes, which can further be split into PP2A, PP2B, and PP2C proteins, assist with cell cycle regulation, dephosphorylation of transcription factors, T-cell activation and deactivation, and signal transduction pathways (Wera and Hemmings, 1995). Numerous other phosphatases are being discovered and characterized, such as the protein currently being studied. Therefore, this list is certainly not exhaustive.

Both phosphatases and kinases can be divided into three groups based on target-site specificity. The first group is serine/threonine-specific; these enzymes catalyze the phosphorylation or dephosphorylation of serine and threonine protein residues. The second is tyrosine-specific, and these catalyze phosphate transfers at tyrosine residues only. The third and rarest group is made up of those enzymes which can catalyze transfers at all three residues; these are most commonly termed dual-specific, an example

of which is the cell cycle regulatory protein cdc25. The phosphatase in this study, found in the periplasm of *Anabaena* PCC 7120, falls under this category as well. The next section deals with circadian rhythms and clocks; one such biorhythm may dictate the enzymatic activity in the unknown phosphatase. Conversely, the phosphatase may regulate the biorhythm, but neither hypothesis has been confirmed.

Circadian Rhythms

The simplest description of a circadian rhythm is any animal behavior which corresponds to the 24-hour cycle of night and day caused by the Earth's rotation. Sleep cycles are prime examples, and many have experienced the discomfort of jet lag, a result of upsetting the internal clock after a long flight. A circadian clock has three necessary components: an oscillator (which is the clock itself), a mechanism for setting the clock to the right time, and mechanisms which relay clock information to other parts of the cell affected by the clock (Golden et al., 1997). Locomotion activity in *Drosophila* has been linked to detection of light by the fly. A second rhythm seen in fruit flies is a peak level of adult emergence from pupae in times of complete darkness. Exact numbers might differ from day to day, but an overall preference for hatching at night is the general rule of thumb for *Drosophila* (Hartl and Jones, 1998).

As complex as rhythms at the level of the organism may seem, those at the molecular level are much more intricate and multifaceted. Regulation of the *Drosophila* genes *per* and *tim* displays a characteristic pattern of activity called a transcriptional feedback network, shown in the illustration below.

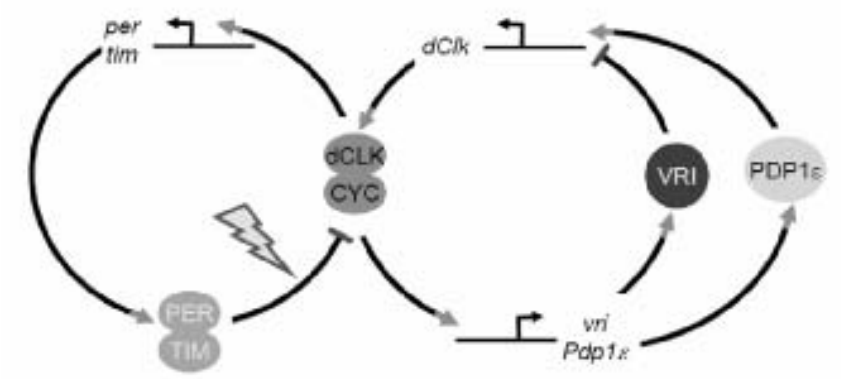

Illustration courtesy of Zoltan Szallasi et al..

In this pathway, the time of day dictates which of these feedback loops is active, and it is a pathway shared in many organisms, including classic models such as the fly and the mouse (Szallasi et al., 2006). The circadian rhythm that is hypothesized to govern the unknown dual-specific phosphatase in *Anabaena* may follow this feedback mechanism, and if so, the phosphatase may occupy the key central position linking multiple pathways together.

Cyanobacteria are some of the simplest organisms which possess circadian rhythm pathways. This group of organisms has the unique ability to both photosynthesize and fix nitrogen. A problem with this system is that nitrogenase, the main enzyme for nitrogen fixation in cyanobacteria, is inactive in the presence of oxygen, and oxygen is a main by-product of photosynthesis. In the 1980s, the separation of nitrogen fixation and photosynthesis in cyanobacteria was studied extensively, and it was discovered that some species fix N_2 at night, when nitrogen stores have been depleted by extensive daytime photosynthetic metabolism. Hypotheses speculating about a circadian rhythm in cyanobacteria naturally followed suit (Golden et al., 1997). Experimentation has since led to the discovery of the clock proteins KaiA, KaiB, KaiC, and SasA in the cyanobacterium *Synechococcus*, with likely homologs in other species of blue-green algae like *Anabaena*.

One proposed mechanism is as follows: early in the day, small KaiA-containing complexes and small KaiC complexes accumulate and positively regulate the transcription of the *kaiBC* gene. Later in the day, buildup of KaiB and KaiC leads to the association of KaiA, KaiB, and SasA with the small KaiC complex, and this larger macromolecular association acts as a repressor of the *kaiBC* gene. Decreasing availability of KaiA and KaiB leads to the dissociation of the large complex, and the cycle repeats itself when the smaller complexes again up-regulate the *kaiBC* gene (Kageyama et al., 2003). The entire process is displayed in the illustration below.

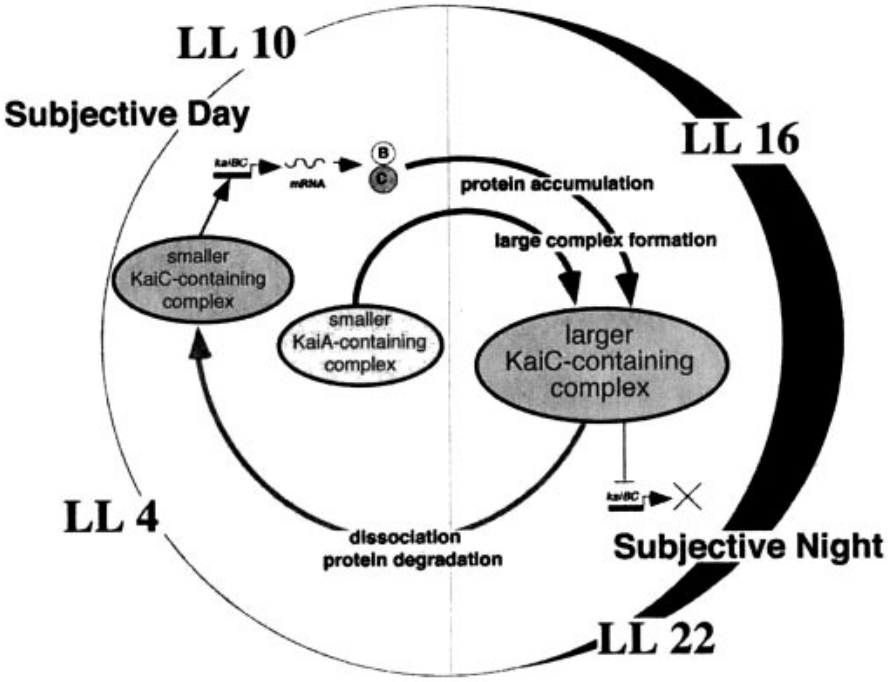

Illustration by Hakuto Kageyama et al..

The gene *pex* has been characterized since the 1980s as well. Its product deals with the clocks timing and output; deletion shortens the circadian rhythm and overexpression lengthens it. Other genes involved with the cyanobacterial clock include *rpoD2* and *ndh2*, but these are not yet well characterized. *cpmA* is yet another gene which controls the output pathway of the clock by regulating yet another group of genes called

psbAI and *psbAII*. Specifically, *cpmA* controls the phase angles of these two genes and keeps them synchronized (Katayama et al., 1999). Another set of studies led to the conclusion that the KaiC-dependent circadian rhythm was a critical mechanism in cell division, but not vice-versa. Genetic manipulation leading to disruption of cell division did not stop the circadian clock, and therefore the clock is independent of the cell cycle (Mori and Johnson, 2001).

Overview of Cyanobacteria

All species of cyanobacteria, or blue-green algae, are prokaryotic, meaning that the individual cells contain no true nucleus or organelles. These organisms synthesize chlorophyll a and a pigment called phycocyanin, which accumulates under certain conditions and gives the algae a namesake bluish hue along with the green color caused by the chlorophyll. Recently, species originally labeled as cyanobacteria were discovered to produce chlorophyll b along with chlorophyll a and no phycocyanin, but they are still grouped together under the blanket name of cyanobacteria (Whitten and Potts, 2002). Cyanobacteria are either unicellular or colonial in nature and can form chains or filaments of cells. One of these cells differentiates into what is known as the heterocyst (the glowing cells in the picture of the next section), a very thick-walled cell where nitrogen fixation occurs. This process is a defining property of cyanobacteria, and it gives cyanobacteria a major growth advantage over eukaryotic algae colonies, which do not fix nitrogen, when nitrogen levels in the environment are low enough to initiate competition. Differentiation is a curious process which occurs when nitrogen levels drop in the habitat of the colony (Whitten and Potts, 2002).

As mentioned earlier, cyanobacteria have the ability to differentiate into a specialized nitrogen-fixing cell called a heterocyst when levels of nitrate and ammonium in the environment are low. The cell has the ability to maintain low oxygen levels so that nitrogenase molecules can perform at maximum efficiency. A secreted glycolipid layer around the cell wall during differentiation ensures minimum gas exchange so as to avoid oxygen contamination. These cells do not possess photosystem II elements and cannot photosynthesize; they also have no ribulose-1,5-bisphosphate carboxylase/oxygenase, or Rubisco, and therefore cannot fix carbon dioxide. The heterocyst receives sugar from the surrounding non-differentiated, or vegetative, cells and in turn supplies the vegetative cells with amino acids and other forms of fixed nitrogen (Wang, 2005).

Multiple gene sets govern the differentiation process which changes a vegetative cell into a heterocyst. These genes can be divided into three groups. The first set consists of those genes which detect environmental nitrogen levels or signals from plant cells. The second set of genes initiates the act of differentiation, and the third set encodes the proteins necessary for heterocyst function (Wang, 2005). The detection gene set includes *ntcA*, whose protein product is a transcription factor which regulates the transcription of genes like *xisA* and *hetR* (Wei et al., 1994). *hetR*, in turn, is the main initiator gene for cyanobacteria. The HetR protein activates the *hetP* and *hetC* genes, which leads to differentiation, and when HetR associates with PatA, it up-regulates its own transcription. Copper ions are also required to activate *hetR* (Buikema and Haselkorn, 2001). Once the heterocyst is formed, two different glycolipids are excreted from the cell and make up the protective shell, one of which is controlled by the activities of the protein phosphatase PrpJ (Jang et al., 2007). Finally, transcription of the *nif* genes completes the

differentiation process; these are the main heterocyst function regulators, and once they are active, the *hetR* gene is shut off by HetN and PatS, which are transcribed after their genes are up-regulated by the *nif* cascade (Buikema and Haselkorn, 2001).

Blue-green algae are considerably robust and hardy, and can endure through multiple types of disturbances. Cyanobacteria show remarkable resistance to radiation, water perturbation, and desiccation, and many have attributed the resistance property to efficient DNA repair. Species survival can also be attributed to interactions between cyanobacterial species and other organisms, most of which are plants. Certain plant species release a chemical which induces the algae to form what is known as hormogonia. These structures are the infecting agents, and they are attracted to the plant by the same chemical which induced its formation. Plants and cyanobacteria enter symbiotic relationships due to the desirability of the algae's nitrogen fixing capability (Whitten and Potts, 2002).

Within the past twenty years, numerous studies on the existence of cyanobacterial species in extremely polluted water have been undertaken, especially after a large bloom of cyanobacteria formed around a massive oil spill in the Persian Gulf in 1991. The first study examined the algae's detoxification of hydrocarbons, and it resulted in the presentation of data pointing to hydrocarbon utilization in some cyanobacterial species. In the mid-1990s, several observational experiments showed that certain species of cyanobacteria, including *Anabaena*, were present at high concentrations in Asian rice paddies, where high amounts of pesticides are used regularly to fend off insects and parasites. Even the herbicides normally used in conjunction with pesticide application had little effect on cyanobacterial growth. A follow-up laboratory study displayed that

Anabaena and *Nostoc* species broke lindane, the common agent in pesticides, down to safer intermediates. *Anabaena*, however, required nitrates in the environment to break up the compound, and all species required a light source to drive the reaction (Dennis et al., 1999).

Nowadays, cyanobacteria are desirable to use in the cleanup of pollutants for several reasons. First, these organisms cost much less to cultivate that heterotrophic alternatives because the algae can metabolize both nitrogen and carbon sources. Second, pollutants are degraded to extremely low levels due to the aforementioned dual-metabolic nature of cyanobacteria. Third, cyanobacteria can be used in both aerobic and anaerobic situations because the heterocyst is completely anaerobic. Finally, the cyanobacteria biomass itself can be recycled through biotechnology. The end result is a cleaner, faster, more efficient anti-pollutant (Dennis, 1999).

However, not all properties of cyanobacteria are helpful to mankind. The same type of blooms and mats formed by colonies of some species of cyanobacteria to break down pollutants can be extremely harmful when formed by other species. The detriment to the animal world is the release of neurotoxins, skin irritants, and hepatotoxins that are both resilient and water-soluble upon cell death. A prime example of just how dangerous these toxins are occurred in 1996, when a hemodialysis center's patients developed liver failure from cyanobacterial toxins present in the water used for the dialysis process. One specific example of a toxin produced is a family of poisons called microcystins, which are natural inhibitors of serine/threonine phosphatases. As can be seen from the section on phosphorylation, a lack of phosphatase activity results in hyperphosphorylation of proteins and rampant growth, which explains the tumors found in the livers of patients

suffering from a microcystin infection. A second toxin used by cyanobacteria is cylindrospermopsin, which binds to DNA and interferes with protein synthesis (Lichtfouse et al., 2005).

Anabaena PCC 7120

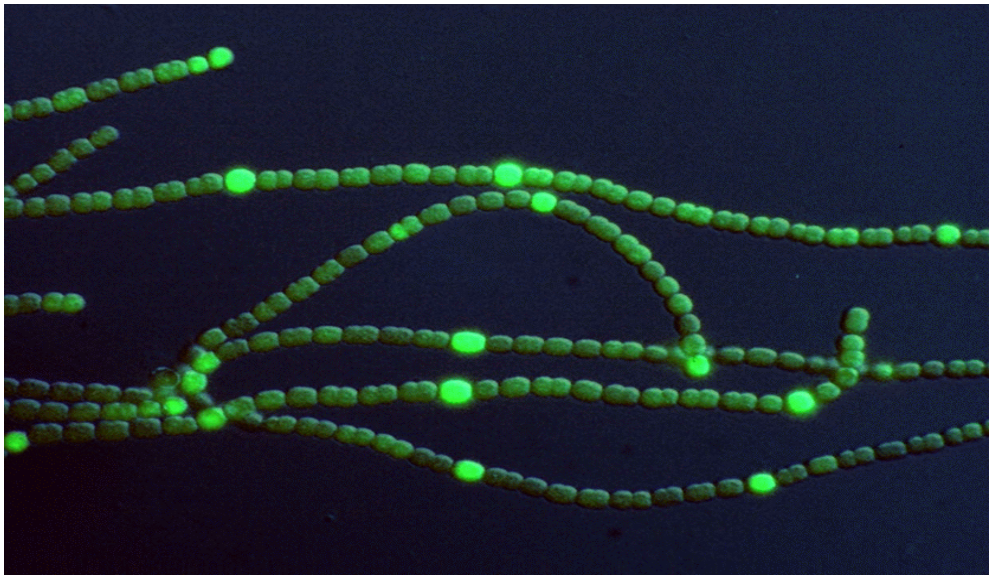

Photo Courtesy of Martin E. Mulligan

Anabaena PCC 7120 is pictured above. The individual cells form longer filaments with multiple heterocysts per strand. This species possesses all of the characteristics of typical cyanobacteria with the following species-specific features. The organism's 6 Mb genome was completely sequenced in 2001, and this opened up a world of information to researchers. The circadian clock proteins KaiA, B, and C were pinpointed, the heterocyst genes listed above were placed, regulatory protein sequences were hypothesized, and protein-coding data was presented. One observation that jumps off the pages of the report is the fact that the circular genome contains 2.5 times the number of components of signal transduction systems as its close relative, *Synechococcus*, and only a 37% sequence

similarity. This serves to illustrate divergence among the different species of cyanobacteria (Kaneko et al., 2001).

One particular abnormality about this species of *Anabaena* is the high number of adenylate cyclase genes present on the genome as compared to other prokaryotes. At least five different genes were discovered, through the use of negative mutants, which code for cyclases. In contrast, *E. coli* produces one, *R. meliloti* produces at least three, and *Stigmatella aurantiaca* possesses two. Cellular functions for these cyclases include regulation of cAMP levels and signal transduction (Katayama and Ohmori, 1997).

Multiple phosphorylation studies have also been completed on PCC 7120. One such experiment led to the characterization of the protein product of a phosphatase gene called *prpA* which possessed the same catalytic domain as PP1, PP2A, and PP2B, all mentioned earlier in this report; curiously, a kinase gene labeled *pknE* exists a mere 300 base pairs from the *prpA* gene. Initial hypotheses indicate that these two enzymes may be regulated together as a unit to ensure the correct level of phosphorylation in the cell (Zhang et al., 1998). A second genomic observational study was done in 2002, but unlike the sequencing experiment, this study looked through the genomic library to find kinase, phosphatase, and regulatory protein sequences. One result relevant to this paper was the finding of multiple unknown or hypothetical protein sequences which possessed similarities to other known proteins. Only one of those hypothesized contained tyrosine phosphatase active-site sequence. The enzyme which is the subject of this paper may be that unknown tyrosine phosphatase. Further experimentation is needed to test that suggestion, however (Wang et al., 2002).

Three of the components of the tyrosine phosphorylation/dephosphorylation network in PCC 7120 were discovered in 1997. One phosphorylated protein was detected with immunoblots when a high level of photons was included in the environment, while a second protein was found phosphorylated in low-photon conditions (McCartney et al., 1997). These two may be related to both the circadian rhythm clock proteins and the unknown phosphatase in the periplasm, but again, that hypothesis is untested.

Previous Research

Research in 1997 by Dr. L. Daniel Howell found both tyrosine kinases and tyrosine-phosphorylated proteins. Using this basis and correctly inferring that a tyrosine phosphatase must exist, he discovered the dual-specific phosphatase currently under investigation. Dephosphorylation activity was detected at optimal pH values in the neutral pH zone, and then the dual-specific nature of the protein was elucidated by observing that both RCML and casein were dephosphorylated in its presence. The phosphatase was sized around 38 kDa using sucrose gradient density ultracentrifugation, and the isoelectric point was experimentally determined to be approximately 6.5. Activity was detected at temperatures as high as 70° C, and cellular partition experiments placed the protein solely in the periplasm of PCC 7120. Finally, light-dependent rhythmic activity was observed using different growth conditions (Howell, 1997).

Characterization of the Novel Dual-Specific Phosphatase

Procedures.

In the spring of 2007, the bioreactor for growing and harvesting the *Anabaena* cells was built and sterilized. It consisted of a large glass jar which could hold approximately 2.25 liters, a rubber stopper to give the reactor an airtight seal, one inlet

tube, and two outlet tubes. A freeze-dried pellet of *Anabaena* PCC 7120 cells was hydrated and used to form a starter culture in BG-11 media (18 mM $NaNO_3$, 230 μM K_2HPO_4, 304 μM $MgSO_4 \cdot 7H_2O$, 245 μM $CaCl_2 \cdot 2H_2O$, 31 μM citric acid, 20 μM ferric ammonium citrate, 3.4 μM EDTA, 240 μM $NaCO_3$, as well as 1 mL trace metal mix per liter of water). This starter culture was then added to the bioreactor along with BG-11 media to a volume of two liters, and the sealed and sterilized glass jar was kept between 25 and 30 degrees Celsius in an incubator. The incubator's lights were also set on a twelve-hour light/dark cycle to simulate natural light; this process was necessary to recreate circadian rhythms in the cell. Filter-sterile air was blown into the jar through the intake tube to supply the cells with fresh air constantly.

For the rest of the spring semester and through the fall semester, cells were harvested every week by stopping the air flow and siphoning the media out of the jar. The cells were centrifuged in conical tubes at 2500 RPM for approximately one minute; the compressed cell pellets were collected into a single tube and centrifuged again to super-concentrate the cells. Optical densities at 600 nm were observed for each collection, and then the harvest was stored in a freezer. The jar was refilled with fresh BG-11 media after each collection stage.

In the fall of 2007, the first biochemical tests were performed on the frozen cell cultures which had been compiled over the last year. One culture which was grown in complete darkness and one culture grown in 24-hour light were thawed and resuspended in a resuspension buffer (20 mL 50 mM Tris, pH 7.4, 0.2 mL 1 mM EDTA, 0.2 mL 1 mM PMSF), and then the cell suspensions were refrozen at -80° C for five minutes. Next, the frozen cells were thawed out again, and then 3 grams of sucrose and a pinch of

lysozyme were added to each mixture. After incubating at room temperature for five minutes, the solutions were centrifuged for fifteen minutes at 4500 RPM. The supernates, both blue to purple in color, were collected and stored on ice. These collections contained the periplasm from the cell cultures, which easily separate from the cells themselves through the freeze-thaw cycles mentioned above. A Bradford protein assay was performed on each sample to determine relative levels of protein inside the periplasm, followed by a time-course enzyme assay to determine overall phosphatase activity.

For the enzyme assay, four assay samples were made from each of the supernate collections from the previous step. Each were prepared using the same formula (0.5 mL 100 mM pNPP, 0.1 mL periplasm extract, 0.1 mL 200 mM Tris, pH 7.4, 0.05 mL 1M $MgSO_4$, 0.25 mL diH_2O). A fifth tube was also prepared using the same formula above except for the extract; this tube served as a zero-time sample. The periplasm was added last, and then all of the assay samples were incubated at 30° C. At five, ten, twenty, and forty minutes, a sample for both the complete-darkness extract and the 24-hour light extract was removed from the incubator. The reaction was stopped by adding 0.1 mL NaOH, and then the samples were scanned in a spectrophotometer at 410 nm to detect how much pNPP (*para*-nitrophenol phosphate) had been converted to pNP (*para*-nitrophenol).

A final test was performed to determine the size of the proteins in the extract. A sample from each extract along with protein standards were loaded into the wells into a pre-cast SDS-PAGE gel and run at standard voltage for 1 hour.

In the spring of 2008, a second set of tests were performed on a culture grown on a 12-hour light/12-hour dark cycle. A 125-mL periplasmic extract was collected, and then

a Bradford protein assay and an enzyme assay were performed. This time, buffers with different pH values were used in five different samples in place of the 200 mM Tris from the previous procedures to determine optimal pH values for phosphatase activity in the periplasm of *Anabaena* PCC 7120. The five buffers used for the assay were acetate (pH 5), citrate (pH 6), Tris (pH 7 and 8), and glycine (pH 9). All five buffers had a concentration of 200 mM. All five samples were incubated at 30° C for five minutes, and then each reaction was stopped with 100 mL NaOH. Spectrophotometric analysis was performed at 410 nm. A size determination was not attempted at this time.

Results.

The dark sample from the fall of 2007 had a protein concentration of 7 mg/mL, and the results of the time-course assay are shown below in Figure 1.

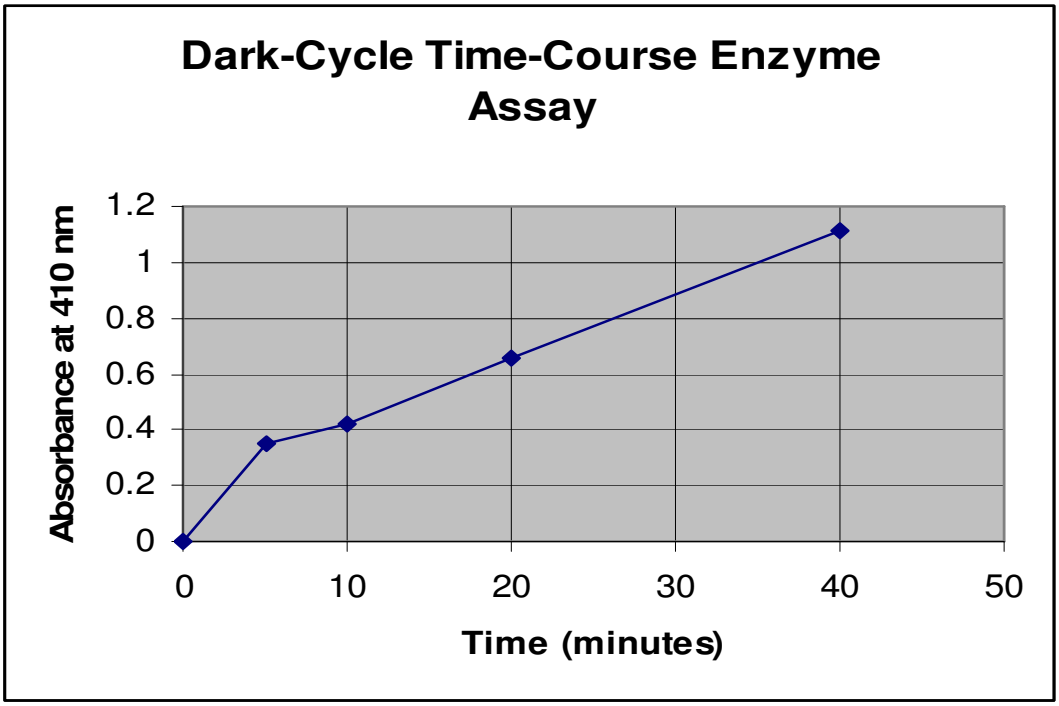

Figure 1. Time-course assay to determine enzyme activity based on reaction time. Readings were made at 5, 10, 20, and 40 minutes. Sample used was grown completely in the dark. Reactions were performed at 30° C at pH 7.5.

Results for the 24-hour light sample are shown below in Figure 2.

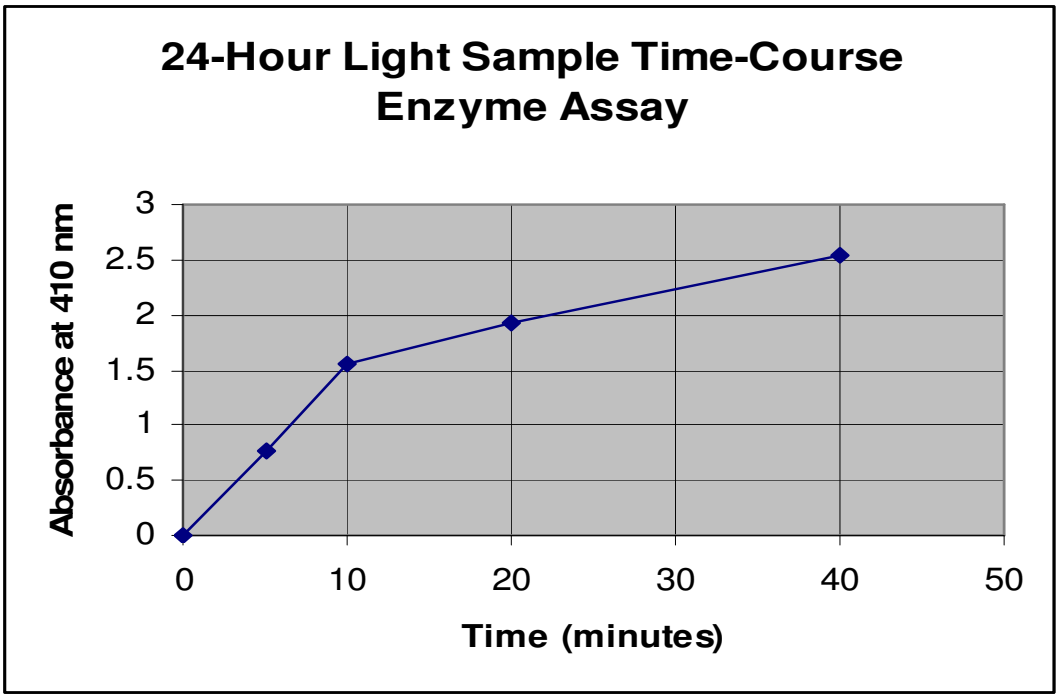

Figure 2. Time-course assay to determine enzyme activity of sample grown in 24-hour light conditions based on reaction time. Readings were made at 5, 10, 20, and 40 minutes. Reactions were performed at 30° C at pH 7.5.

The SDS-PAGE gel was not loaded correctly and thus the data cannot be used to determine the size of proteins in the periplasm.

The 12-hour light/12-hour dark cycle sample had a protein concentration of 3.4 mg/mL, and the results of the optimal pH enzyme assay are shown below in Figure 3.

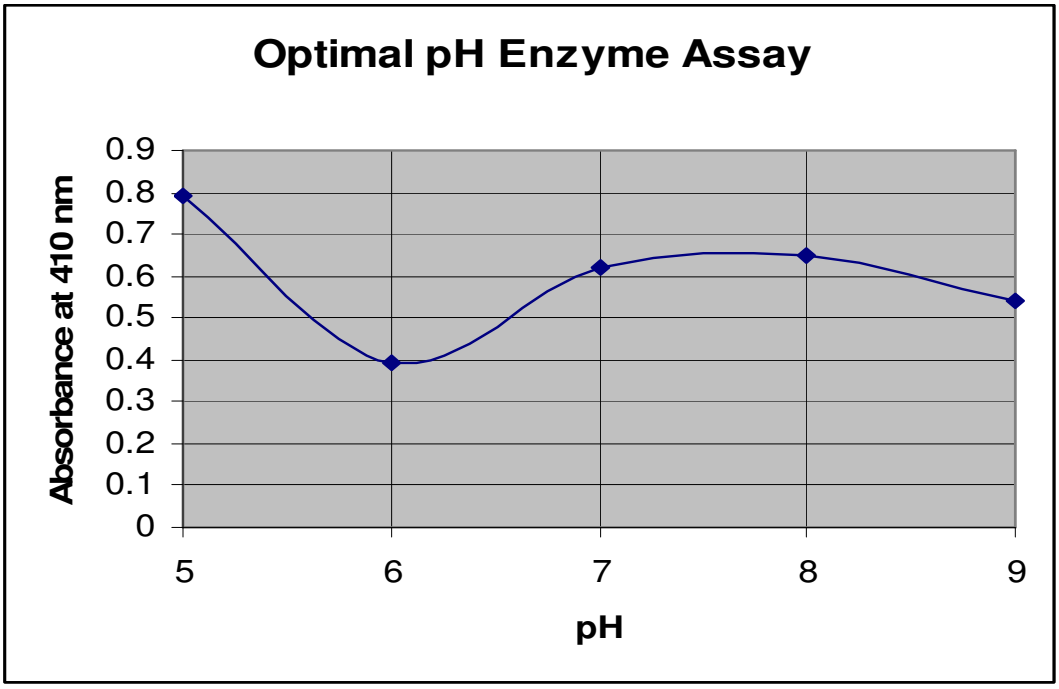

Figure 3. Optimal pH was tested using buffers of different pH values. Reactions were run at 30° C for 5 minutes in 5 buffers of different pH values.

Conclusions.

Comparing the enzyme activities of Figure 1 and Figure 2, phosphatase activity in the periplasm of *Anabaena* PCC 7120 increases dramatically between dark growth and light growth, which is a biochemically expected result for a photosynthetic organism like cyanobacteria. The results for these two samples may very well be the two extreme activity levels for the circadian rhythm present in *Anabaena*, with a high activity seen during the day and a low activity seen at night. There are a few potential causes for this phenomenon. The first is a lower level of gene transcription at night due to repressing agents present in the dark and broken down in the light. A second cause could be a photon-sensitive regulator in the periplasm, and a third possible cause is the presence of an enzymatic inhibitor during the dark hours.

The optimal pH test poses an interesting problem for this experiment. Two different peaks of activity, one around pH 5 and one around pH 7.5, were seen during the assay, which may suggest that the enzyme prefers a wide range of pH values, as opposed to most other enzymes, which prefer a tighter range of pH levels. A second and more likely explanation is the presence of multiple phosphatases in the periplasm, represented by the different activity peaks. Limiting the conclusions here is the fact that only five buffers were used; future work on the enzyme in question needs to narrow down the specific peak occupied by the dual-specific phosphatase, and that will probably involve using different substrates for the reaction.

Future experimentation also needs to include isolation of the enzyme to achieve exact characterization, which is nearly impossible when using only the raw periplasmic extract. After the protein is isolated, the size of the protein can be determined and confirmed, and the protein sequence can be established. Next, the protein sequence can be linked to a chromosomal region by using the genomic sequence elucidated by Kaneko et al.

Other tests to perform could include inhibition studies, confirmation of the optimal pH to determine which peak seen in this experiment belongs to the unknown phosphatase, and determination of the enzyme's natural substrate (or substrates). An interesting study which could yield valuable information would be a correlation experiment between the unknown protein and the known circadian-rhythm proteins KaiA, KaiB, KaiC, and SasA previously mentioned. Positive results there would provide further evidence for the protein's circadian rhythm. Cloning of the gene encoding the protein is also foreseeable in the near future. Researchers might then try to find homologs

in other species of cyanobacteria or even uses for the protein outside its natural habitat. As can readily be seen, this initial study merely provides the framework for further experimentation.

Works Cited

Alessi, Dario R.; Cohen, Philip. "Mechanism of activation and function of protein kinase B." *Current Opinion in Genetics and Development* 8: 55-62. 1998.

Buikema, William J.; Haselkorn, Robert. "Expression of the *Anabaena* hetR gene from a copper-regulated promoter leads to heterocyst differentiation under repressing conditions." *PNAS* 98 (5): 2729-2734. 2001.

Cardone, Michael H.; Roy, Natalie; Stennicke, Henning R.; Slaveson, Guy S.; Franke, Thomas F.; Stanbridge, Eric; Frisch, Steven; Reed, John C. "Regulation of cell death protease caspase-9 by phosphorylation." *Science* 282: 1318-1321. 1998.

Cohen, Philip; Cohen, Patricia. "Protein phosphatases come of age." *Journal of Biological Chemistry* 264 (36): 21435-21438. 1989.

Copeland, Robert Allen. *Enzymes: A Particular Introduction to Structure, Mechanism, and Data Analysis*. New York: John Wiley and Sons, Inc., 2000.

Dennis, P. J.; Godfree, A. F.; Stewart-Tull, D. E. S. *Aquatic Microbiology*. Oxford: Blackwell Science, 1999.

Garcia-Morales, Pilar; Minami, Yasuhiro; Luong, Elise; Klausner, Richard, D.; Samelson, Lawrence E. "Tyrosine phosphorylation in T cells is regulated by phosphatase activity: Studies with phenylarsine oxide." *PNAS* 87: 9255-9259. 1990.

Garrett, Reginald H.; Grisham, Charles M. *Biochemistry, 3rd ed.* Belmont, California: Thomson Brooks/Cole, 2005.

Golden, Susan S.; Ishiura, Masahiro; Johnson, Carl Hirschie; Kondo, Takao. "Cyanobacterial circadian rhythms." *Annual Review of Plant Physiology and Plant Molecular Biology* 48: 327-354. 1997.

Hartl, Daniel L.; Jones, Elizabeth W. *Genetics: Principles and Analysis*. Surdbury, Massachusetts: Jones and Bartlett Publishers, Inc., 1998.

Howell, L. Daniel. *Characterization of IphP from Nostoc commune UTEX 584 and a Dual-Specificity Protein Phosphatase from Anabaena PCC 7120*. Doctoral Dissertation, Virginia Polytechnic Institute and State University, 1997.

Jang, Jichan; Wang, Li; Jeanjean, Robert; Zhang, Cheng-Cai. "PrpJ, a PP2C-type protein phosphatase located on the plasma membrane, is involved in heterocyst maturation in the cyanobacterium Anabaena sp. PCC 7120." *Molecular Microbiology* 64 (2): 347-358. 2007.

Kageyama, Hakuto; Kondo, Takao; Iwasaki, Hideo. "Circadian formation of clock protein complexes by KaiA, KaiB, KaiC, and SasA in cyanobacteria." *Journal of Biological Chemistry* 278 (4): 2388-2395. 2003.

Kaneko, Takakazu; Nakamura, Yasukazu; Wolk, C. Peter; Kuritz, Tanya; Sasamoto, Shigemi; Watanabe, Akiko; Iriguchi, Mayumi; Ishikawa, Atsuko; Kawashima, Kumiko; Kimura, Takaharu; Kishida, Yoshie; Kohara, Mitsuyo; Matsumoto, Midori; Matsuno, Ai; Muraki, Akiko; Nakazaki, Naomi; Shimpo, Sayaka; Sugimoto, Masako; Takazawa, Masaki; Yamada, Manabu; Yasuda, Miho; Tabata, Satoshi. "Complete genomic sequence of the filamentous nitrogen-fixing cyanobacterium *Anabaena* sp. Strain PCC 7120." *DNA Research* 8 (5): 205-213. 2001.

Katayama, Mitsunori; Ohmori, Masayuki. "Isolation and characterization of multiple adenylate cyclase genes from the cyanobacterium *Anabaena* sp. strain PCC 7120." *Journal of Bacteriology* 179 (11): 3588-3593. 1997.

Katayama, Mitsunori; Tsinoremas, Nicholas F.; Kondo, Takao; Golden, Susan S. "cpmA, a gene involved in an output pathway of the cyanobacterial circadian system." *Journal of Bacteriology* 181 (11): 3516-3524. 1999.

Kitazawa, Toshio; Gaylinn, Bruce D.; Denney, Gerald H.; Somlyo, Andrew P. "G-protein-mediated Ca2+ sensitization of smooth muscle contraction through myosin light chain phosphorylation." *Journal of Biological Chemistry* 266 (3): 1708-1715. 1991.

Lichtfouse, Eric; Schwarzbauer, Jan; Robert, Didier. *Environmental Chemistry: Green Chemistry and Pollutants in Ecosystems*. Berlin, New York: Springer Science & Business Media, 2005.

Lipfert, Lorraine; Haimovich, Beatrice; Schaller, Michael D.; Cobb, Bradley S. Parsons, J. Thomas; Brugge, Joan S. "Integrin-dependent phosphorylation and activation of the protein tyrosine kinase pp125^{FAK} in platelets." *Journal of Cell Biology* 119 (4): 905-912. 1992.

Matthews, Harry Roy; Freedland, Richard A.; Miesfeld, Roger L. *Biochemistry: A Short Course*. New York: John Wiley and Sons, Inc., 1997.

McCartney, Bruno; Howell, L. Daniel; Kennelly, Peter J.; Potts, Malcolm. "Protein tyrosine phosphorylation in the cyanobacterium *Anabaena* sp. Strain PCC 7120." *Journal of Bacteriology* 179 (7): 2314-18. 1997.

Mori, Tetsuya; Johnson, Carl Hirschie. "Independence of circadian timing from cell division in cyanobacteria." *Journal of Bacteriology* 183 (8): 2439-2444. 2001.

Newton, Alexandra C. "Protein kinase C: Structure, function, and regulation." *Journal of Biological Chemistry* 270 (48): 28495-28498. 1995.

Reddy, Chada S. "Alterations in protein kinase A signaling and cleft palate: a review." *Human and Experimental Toxicology* 24 (5): 235-242. 2005.

Sakaguchi, Kazuyasu; Herrera, Julio E.; Saito, Shin'ichi; Miki, Toru; Bustin, Michael; Vassilev, Alex; Anderson, Carl W.; Appella, Ettore. "DNA damage activates p53 through a phosphorylation–acetylation cascade." *Genes and Development* 12: 2831-2841. 1998.

Szallasi, Zoltan; Stelling, Jörg; Periwal, Vipul. *System Modeling in Cell Biology: From Concepts to Nuts and Bolts*. Cambridge, Massachusetts: MIT Press, 2006.

Wang, Li; Sun, Yi-Ping; Chen, Wen-Li; Li, Jian-Hong; Zhang, Cheng-Cai. "Genomic analysis of protein kinases, protein phosphatases and two-component regulatory systems of the cyanobacterium *Anabaena* sp. strain PCC 7120." *FEMS Microbiology Letters* 217: 155-165. 2002.

Wang, Yi-Ping. *Biological Nitrogen Fixation, Sustainable Agriculture and the Environment: Proceedings of the 14th International Nitrogen Fixation Congress*. Dordrecht, Great Britain: Springer Science and Business Media, 2005.

Wei, Tai-Fen; Ramasubramanian, T. S.; Golden, James W. "*Anabaena* sp. strain PCC 7120 *ntcA* gene required for growth on nitrate and heterocyst development." *Journal of Bacteriology* 176 (15): 4473-4482. 1994.

Wera, Stefaan; Hemmings, Brian A. “Serine/threonine protein phosphatases.” *Biochemistry Journal* 311: 17-29. 1995.

Whitton, Brian A.; Potts, Malcolm. *The Ecology of Cyanobacteria: Their Diversity in Time and Space*. New York: Kluwer Academic Publishers, 2002.

Zhang, Cheng-Cai; Friry, Aline; Peng, Ling. “Molecular and genetic analysis of two closely linked genes that encode, respectively, a protein phosphatase 1/2A/2B homolog and a protein kinase homolog in the cyanobacterium *Anabaena* sp. strain PCC 7120.” *Journal of Bacteriology* 180 (10): 2616-2622. 1998.

Acknowledgments

I would like to first off thank my thesis advisor, Dr. Daniel Howell, and my thesis committee members, Dr. Mark Hemric and Dr. Emily Heady. Their help and guidance on this project can be described as invaluable to me. I would like to thank the professors who, over the years, have taught me how to learn and how to think critically about projects like this. I would like to thank my family and friends for constant encouragement, including my wonderful fiancée/wife (depending on whether this is read before or after January 3, 2009), Michelle McConnachie, who has provided an endless source of motivation and support throughout it all.