Identification of a Low Molecular Weight Protein Tyrosine Phosphatase and Its Potential Physiological Substrates in "Synechocystis" sp. PCC 6803

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Identification of a Low Molecular Weight Protein Tyrosine Phosphatase and Its Potential Physiological Substrates in *Synechocystis* sp. PCC 6803

Archana Mukhopadhyay

Abstract

The predicted protein product of open reading frame *slr0328* from *Synechocystis* sp. PCC 6803, SynPTP, possesses significant amino acid sequence similarity with known low molecular weight protein tyrosine phosphatases (PTPs). To determine the gross functional properties of this hypothetical protein, open reading frame *slr0328* was cloned, and its predicted protein product was expressed in *E. coli*. The recombinant protein, SynPTP, was purified by metal ion column chromatography. The catalytic activity of SynPTP was examined toward several exogenous protein substrates that had been phosphorylated on either tyrosine residues or serine residues. SynPTP exhibited phosphatase activity toward tyrosine phosphorylated protein substrates ($V_{\text{max}}$ toward phosphotyrosyl $^{32}$P-casein was 1.5 nmol/min/mg). However, no phosphatase activity was detected toward serine phosphorylated protein substrates. SynPTP displayed phosphohydrolase activity toward several organophosphoesters including para-nitrophenyl phosphate (p-NPP), beta-napthyl phosphate and phosphotyrosine but not toward alpha-napthyl phosphate, phosphoserine, or phosphothreonine. Kinetic analysis indicated that the $K_{m}$ (0.6 mM) and $V_{\text{max}}$ (3.2 µmole/min/mg) values for SynPTP toward pNPP are similar to
those of other known bacterial low molecular weight PTPs. The protein phosphatase activity of SynPTP was inhibited by sodium orthovanadate, a known inhibitor for tyrosine phosphatases, but not by okadaic acid, an inhibitor for many serine/threonine phosphatases. Mutagenic alteration of the predicted catalytic cysteine, Cys_7, to serine abolished enzyme activity. Several phosphotyrosine containing proteins were detected from the whole cell extracts of *Synechocystis* sp. PCC 6803 through immunoreactions using anti-phosphotyrosine antibody. SynPTP was observed to dephosphorylate three of these proteins *in vitro*. Two of these proteins were identified by peptide-mass fingerprinting analysis, as PsaD (photosystem I subunit II) and CpcD (phycocyanin rod linker protein). In addition, several phosphotyrosine proteins were detected from the soluble and membrane fractions of *Synechocystis* sp. PCC 6803 cell extracts by *in vitro* substrate trapping as potential endogenous substrates of SynPTP. Two of these proteins were identified as the alpha and beta subunits of phycocyanin. We therefore speculate that SynPTP might be involved in the regulation of photosynthesis in *Synechocystis* sp. PCC 6803.
Dedication

To my beloved little nephew Biboswan Ghosh

who is no more in this world

to light up our lives
Acknowledgements

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<table>
<thead>
<tr>
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<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>α-NP</td>
<td>Alpha napthyl phosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>β-NP</td>
<td>Beta napthyl phosphate</td>
</tr>
<tr>
<td>CAPS</td>
<td>3-(cyclohexylamion)-1-propane sulfonic acid</td>
</tr>
<tr>
<td>cPTP</td>
<td>Conventional PTP</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>DSP</td>
<td>Dual specific phosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethelene diamine tetra acetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethelene glycol-bis(beta-aminoethyl ether)-N, N, N',N'-tetra acetic acid</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric focusing</td>
</tr>
<tr>
<td>IPG</td>
<td>Immobiline pH gradient</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-thiogalactopyranoside</td>
</tr>
<tr>
<td>kbp</td>
<td>Kilobase pair</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>LMW PTP</td>
<td>Low molecular weight protein tyrosine phosphate</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin basic protein</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino) ethane sulfonic acid</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Phycocyanin</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulphonyl fluoride</td>
</tr>
<tr>
<td>pNPP</td>
<td>para-Nitrophenyl Phosphate</td>
</tr>
<tr>
<td>PTP</td>
<td>Protein tyrosine phosphatase</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>RCML</td>
<td>Reduced carboxymethylated maleylated Lysozyme</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate/EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline + Tween 20</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic aci</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>Mr</td>
<td>Relative molecular mass</td>
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Chapter I

Introduction:

Protein Phosphorylation/Dephosphorylation: Nature’s Most Fundamentally Important Cellular Signaling Mechanism:

Living cells must be able to sense external or internal changes in environmental conditions in order to survive. Cells respond to these environmental changes through many cellular signaling processes, which are controlled by interactions between proteins. One of the most important regulatory mechanisms in signal transduction and information processing in cells is reversible protein phosphorylation. This cellular process is one of the preeminent molecular mechanisms for modulating the functional properties of proteins, and it takes place virtually in every living cell (Cozzone, 1988; Stock et al., 1990; Garry et al., 2002).

The covalent attachment of a phosphoryl group, which possesses high charge density and extreme hydrophilicity, to an amino acid side chain on a protein often causes a conformational change in the protein that may alter its functional properties (Westheimer, 1987; Johnson and Lewis, 2001; Kennelly, 2003). Two opposing classes of enzymes, protein kinases and protein phosphatases, regulate the phosphorylation state of proteins. Protein kinases transfer the \( \gamma \) phosphate of a nucleoside triphosphate, generally ATP, to a phosphoacceptor amino acid side chain on a substrate protein. Examples of common phosphoacceptor amino acids include serine, threonine, tyrosine, aspartate and histidine. Phosphoproteins are quite stable under physiological
conditions, but they can be readily restored to their original, unmodified state through the catalytic action of protein phosphatases, which hydrolyze the protein-phosphate bond. This cellular process thus acts as a molecular switch to turn proteins “off” and “on” between functionally distinguishable states (Johnson and Lewis, 2001). The catalytic reactions of a typical protein kinase and protein phosphatase are illustrated below.

![Diagram of catalytic reaction of protein kinase and protein phosphatase]

Figure 1-1. A catalytic reaction of a protein kinase and protein phosphatase.

This mechanism mediates many cellular signaling processes. These include several metabolic pathways (Patel et al., 2004; Boden, et al., 2003; Sampaio et al., 2003), cell division and cell growth (Ye and Zhang, 2004; Ustach et al., 2004; Chiarugi, et al., 2000), gene transcription and translation (Bell et al., 2001; Bhat, 2003), hormonal responses (Gonzalez et al., 2003), neuronal signal
transduction (Kins et al., 2003), muscle contraction (Gorenne, et al., 2004), and pathogenic virulence factors (Liang et al., 2003; Guan and Dixon, 1990; McDonald et al., 2003).

Abnormal protein phosphorylation is a cause or consequence of major diseases such as cancer (Wojciechowski et al., 2003), diabetes (Valdez et al., 2001), Alzheimer’s disease (Pei et al., 2003), and many inflammatory disorders (Stuckey et al., 1994). Defects in genes that encode protein kinases and protein phosphatases underlie a number of inherited disorders including a variety of leukemias, lymphomas, and severe combined immunodeficiency syndromes (Stuckey et al., 1994; Gary et al., 2002).

A Historical Perspective of Protein Phosphorylation:

For many years, scientists questioned whether protein phosphorylation was a global biological regulatory mechanism or was limited to eukaryotic organisms. The regulation of enzyme activity by protein phosphorylation was first demonstrated by Krebs and Fisher during their studies of glycogen metabolism in skeletal muscle (Krebs and Fisher, 1956). Their findings showed that phosphorylase kinase phosphorylates glycogen phosphorylase and converts it to its active form. Due to its early association with hormone action, protein phosphorylation/dephosphorylation was considered the molecular expansion of the neuroendocrine system into the interior of cells (Shi et al., 1998a). Initially, it was widely believed that regulatory protein phosphorylation arose as a late evolutionary response to the specific needs of higher eukaryotes, targeting the
hydroxyl side chains of serine, threonine and tyrosine (Greengard, 1978). The existence of regulatory protein phosphorylation in bacteria was first discovered in late 1970s by Garnak and Reeves (Garnak and Reeves, 1978). They observed in *E. coli* that the catalytic activity of isocitrate dehydrogenase was regulated by phosphorylation of a serine residue. In mid-1980s, the discovery of the histidine protein kinases of the bacterial two-component system, which phosphorylate aspartate residues, established that protein phosphorylation was a global biological regulatory mechanism (Stock et al., 1989; Bourret et al., 1989; Egger et al., 1997).

**Signal Transduction: Eukaryotes versus Prokaryotes:**

Cellular signal transduction processes operate through the action of protein kinases and protein phosphatases in both prokaryotes and eukaryotes. These actions result in modification of gene expression or enzymatic reactions that enable cells to generate an appropriate response to a given signal (Zhang, 1996) and are often transduced from cell membrane to intracellular targets (Garry et al., 2002).

In multicellular organisms, a signal is often transmitted through a cascade of protein phosphorylation/dephosphorylation events that control a vast array of physiological processes. This is perhaps best understood from studies of mitogen activated protein kinases (MAPK), which comprise a family of protein kinases whose functions and regulation have been conserved during evolution from unicellular eukaryotic organisms such as yeast to multicellular eukaryotes.
including humans. These pathways generally contain three protein kinases, which function in the following order: a MAP kinase kinase kinase (MAPKKK or MEKK or Raf) phosphorylates MAP kinase kinase (MAPKK or MEK), which in turn phosphorylates MAP kinase (MAPK) (Gustin et al., 1998). Many serine/threonine phosphatases or protein tyrosine phosphatases interact with this pathway by dephosphorylating the protein kinases of the MAPK cascade or downstream MAPK phosphoprotein substrates. MAPKs phosphorylate specific serine and threonine residues of target protein substrates and regulate cellular activities ranging from gene expression, mitosis, movement, metabolism, and programmed cell death (Garry et al., 2002; Chiarugi et al., 1997; Riggaci et al., 2001; Chiarugi et al., 2000; Fiaschi et al., 2001). These extracellular signal-regulated kinases function in the control of cell division. Inhibitors of these enzymes are being explored as potential anticancer agents. The p38 MAPKs are activated by inflammatory cytokines and environmental stresses and may contribute to diseases like asthma and autoimmune responses (Stuckey et al., 1994).

Many prokaryotic organisms employ a two-component regulatory system to sense the changes in their environment. This two-component system is characterized by phosphotransfer reactions involving histidine and aspartate residues in highly conserved signaling domains. Two proteins are involved in this pathway, a histidine protein kinase, also called a sensor kinase, and a response regulator. The N-terminal portion of the histidine kinase functions as an input domain, detecting environmental stimuli directly or interacting with an upstream
receptor. The C-terminal end of the protein undergoes autophosphorylation on a conserved histidine residue. The response regulator has a receiver domain at its N-terminus, which catalyzes transfer of the phosphoryl group from the histidine residue of sensor kinase to a conserved aspartate residue of response regulator. The phosphorylation of the receiver domain of a response regulator causes it to bind target proteins and elicit an adaptive response to the stimulus (Appleby et al., 1996; Perraud et al., 1999). The two-component signaling pathway is utilized for responding to a wide array of environmental stimuli, including changes in osmolarity, nutrient availability, and host proximity.

Current evidence suggests that “bacteria like” two-component regulatory systems are present in many eukaryotic organisms such as yeast (Ota and Varshavsky, 1993, Maeda et. al, 1994, Tao et. al, 1999); the fungi Neurospora crassa and Candida albicans (Alex et al. 1996; Calera et.al, 1998); the slime mold Dictyostellium discoideum (Wang et. al, 1999); and the plant Arabidopsis thaliana (Chang et al, 1993). Similarly, homologs of eukaryotic protein kinases and protein phosphatases have been identified in many prokaryotic organisms. Endogenous protein serine/threonine kinase activity was first detected in the bacteria S. typhimurium (Wang and Koshland, 1978) and E. coli (Manai and Cozzone, 1979). Other examples include the protein tyrosine kinases Ptk in Acinetobacter johnsonii (Grangeasse et al., 1998) and Wzc in E. coli (Vincent et al., 1999). These endogenous kinases autophosphorylate at multiple tyrosine residues, which are dephosphorylated by a protein tyrosine phosphatase, Ptp, from A. johnsonii and Wzb from E. coli, respectively.
Although eukaryotic and prokaryotic protein phosphorylation networks maintain certain fundamental distinctions, evidences suggested that these systems and their corresponding enzymes share a number of structural and functional similarities. Conclusive evidences suggested that phosphorylation/dephosphorylation network existed before the divergence of prokaryotes and eukaryotes and arose early in evolution as a dynamic and versatile regulatory mechanism (Kennelly and Potts, 1996; Zhang, 1996). Since prokaryotes are simple, non-complicated, and genetically malleable, it is easy to study “eukaryotic-like” protein kinases and protein phosphatases in prokaryotes. Thus this study serves as a powerful set of tools to understand the mechanism of signal transduction in both prokaryotes and eukaryotes.

Classification of Protein Phosphatases:

In eukaryotes, five superfamilies of protein phosphatases have been identified. They are the PPP and PPM families of protein serine/threonine phosphatases, and three families of protein tyrosine phosphatases (PTP), which are the conventional PTPs, low molecular weight PTPs, and Cdc 25 family (Fauman and Saper, 1996; Ramponi and Stefani, 1997). Some conventional PTPs and the phosphatases of the Cdc25 family are referred to as dual specific protein phosphatases (DSPs), because they hydrolyze both the aryl phosphomonoester of phosphotyrosine and the alkyl phosphohomonoester linkages of serine and threonine (Raugei et al., 2002).
The members of PPP superfamily in eukaryotes are metalloenzymes, which strongly bind a pair of metal ions adjacent to each other, probably Fe^{3+} and either Mn^{2+} or Mg^{2+} (Cohen, 1997). The most prominent members of this family are PP1, PP2A, and PP2B. The eukaryotic members of PPM superfamily include PP2C and pyruvate dehydrogenase phosphatase. PPMs are dependent on exogeneous metals to support catalytic activity.

Among the three protein tyrosine phosphatase (PTP) superfamilies, little sequence similarity exists. Three-dimensional structural data show that these superfamilies share conserved structural elements, namely a phosphate binding loop, which contains PTPase signature motif VC(X)_5RS/T and an essential aspartate that serves as a catalytic acid/base (Zhang, 1998; Kennelly, 2001). In prokaryotes, four of the five superfamilies of protein phosphatases have been encountered so far (Kennelly, 2002). The presence of Cdc25 family in prokaryotes has not been established, however, the sulfurtransferase rhodanese, a homologue and likely precursor of Cdc25, is present in many bacteria (Fauman et al., 1998).

Structure of Protein Tyrosine Phosphatases (PTPs):

Active site sequences:

PTPs differ in the location of the active site sequence C-X_5-RS/T, and conserved aspartate residue within the sequence of catalytic domain. Unlike the PPP or PPM superfamilies, PTPs do not require metal ions for catalysis (Zhang et al., 1998a). In the conventional PTPs the active site sequence resides in the
central portion of the catalytic domain, which contains approximately 230 amino acids. The residue preceding the catalytic cysteine is histidine (HC-X$_5$-RS/T). The conserved aspartate is located 25 to 45 residues to the N-terminal side of the catalytic cysteine (Fauman et al., 1996). The active site sequence motif of Cdc25 family or other DSPs (VHR or VH1 from mamalian) closely resembles to that of conventional PTPs, D-X$_{=45}$-CX$_5$RS/T (Kennelly, 2001; Zhang, 1998). In the low molecular weight (LMW) PTPs, the active site sequence is located near the extreme N-terminus (usually 5~10 residues) of the catalytic domain ($\approx$150 residues), and is usually preceded by either a valine or a leucine residue (L/VC-X$_5$-RS/T). The conserved aspartate is located 85 to 105 residues to the C-terminal side of the catalytic cysteine and is followed by a proline (Ramponi et al., 1997). The active site sequences of three PTP superfamilies are illustrated in Table 1 (adapted from Kennelly, 2001).

<table>
<thead>
<tr>
<th>PTP superfamily</th>
<th>Prominent members</th>
<th>active site sequence motifs</th>
</tr>
</thead>
<tbody>
<tr>
<td>cPTP</td>
<td>PTP1B, YopH</td>
<td>WPD-X$_{=45}$-HCX$_5$RS/T</td>
</tr>
<tr>
<td>Cdc25</td>
<td>Cdc25 A, B, C</td>
<td>D-X$_{=45}$-CX$_5$RS/T</td>
</tr>
<tr>
<td>LMWPTP</td>
<td>Wzb, Mtp</td>
<td>L/VC-X$<em>5$-RS/T-X$</em>{85-105}$DP</td>
</tr>
</tbody>
</table>

CPTP denotes conventional PTP.

Three-dimensional structure of PTPs:

The three-dimensional structures of two cPTPs (PTP1B, Barford et al.,
1994, Jia et al., 1995; Yersinia PTP, Stuckey et al., 1994, Fauman et al., 1996), a DSP (VHR from human, Yuvaniyama et al., 1996), and several low molecular weight PTPs (Bovine heart phosphatase, Su et al., 1994; Zhang et al., 1994a; Mptp from \textit{M. tuberculosis}, Madhurantakam, et al., 2005; Ltp1 from \textit{S. cerevisiae} Wang et al., 2000) have been determined. The structural topology of these three PTP superfamilies are distinctly different from each other but, interestingly, they share a similar active site (phosphate binding site, CX$_5$RS/T) architecture (Zhang, 1998; Denu et al., 1996). The PTPs (cPTPs, DSPs, and LMW PTPs) consist of a single $\alpha$$+$$\beta$ type domain where four parallel $\beta$-strands form a core structure and are flanked by $\alpha$ helices. The residues of the PTP signature motif, CX$_5$RS/T, of cPTPs, DSPs, and LMW PTPs form a loop that is referred to as the P-loop. Figure 1-2 represents the P-loop structure of three PTP superfamilies.

This P-loop is located between the $\beta$-turn at the COOH terminus of the $\beta$-strand that ends with HC or VC and the first turn of an $\alpha$-helices, which starts with RS (Figure 1-3).
Figure 1-3. A sequence alignment of low molecular weight PTPases from yeast (LTP1) and bovine heart (BPTP). Identical residues are highlighted in gray. Asterisks indicate the active site sequence residues and conserved acid/base residue (Asp). Locations of P-loop, variable loop, and movable loop are shown as indicated in red. Above the sequences are listed the secondary structure elements of LTP1 (Wang et al., 2000).

The catalytic cysteine of the signature motif resides at the base of the P-loop (Zhang, 1998; Zhang et al., 1998a). The guanidium group of arginine points in toward the active site where it assists in phosphate binding and the catalytic
reaction (Denu et al., 1996; Zhang, 1998; Wang et al., 2000). A variable loop, which contains a group of hydrophobic residues, forms one wall and the floor of the active site cavity (Zhang et al., 1998a). The loop containing the catalytic aspartate, known as the movable loop, forms the other wall of the cavity (Wang et al., 2000; Denu et al., 1996; Jia et al., 1995). The catalytic Asp is located at opposite of the catalytic cysteine and is pointed toward the bound oxyanion of the phosphorylated substrate (Stuckey et al., 1994, Zhang et al., 1994, Su et al., 1994). It was observed in the unliganded (no bound substrate) Yersinia PTPase (YopH) structure that Asp$^{356}$ is greater than 10Å from the P-loop. In the ligand-bound (substrate bound) structure, this movable loop containing Asp$^{356}$ moved like a “flap” to cover the active site (Stuckey et al., 1994; Zhang, 1998). Figure 1-4 illustrates the P-loop, variable loop, and movable loop of a human low molecular weight PTP, HCPTPA (Zhang et al., 1998a).
Figure 1-4. A ribbon diagram of the fold of a human low molecular weight PTP (HCPTPA). The active site sequence (P-loop) is located in the segment connecting the β1 strand and the first turn of α1 helix. Variable loop resides between the β2 strand and α2 helix. The movable loop, containing conserved Asp, is present in the region connecting β4 strand and α5 helix (Zhang et al., 1998a).

The binding of the tyrosine ring of the substrate and substrate selectivity of the enzyme are mainly determined by the depth of the active site cavity and the identity of the residues lining the walls of the cavity (Zhang et al., 1998a; Zhang et al., 1994a; Denu et al., 1996). The variable loop also contributes to the depth of the active site cavity (Zhang et al., 1998a; Zhang, 1998). In DSPs (VHR), no sequence homology is apparent between the variable loop and that of either cPTPs (PTP1B) or low molecular weight PTPs (bovine heart PTP). In VHR, the variable loop is 75% shorter than that of PTP1B, which leads to a decrease of the overall depth of binding pocket (Denu et al., 1996; Stuckey et al., 1994; Yuvaniyama et al., 1996). It is believed that the primary difference between
phosphotyrosine specific or dual specific PTPs is the depth of the binding pocket. The shallow and wide active site pocket (6Å depth) (Yuvaniyama et al., 1996) of a DSP (VHR) can accommodate both phosphotyrosine and phosphoserine/phosphothreonine. Due to its deep and narrow binding pocket (9Å depth) of a PTP (PTP1B, YopH), (Stuckey et al., 1994; Barford et al., 1994) only phosphotyrosine moiety can reach the catalytic cysteine at the base of the active site crevice of a PTP (Yuvaniyama, et al., 1996; Denu et al., 1996; Zhang et al., 1998a).

Mechanism of a PTP:

All PTPs share a two step ping-pong mechanism. First, the catalytic cysteine carries out a nucleophilic attack on the phosphoester of the protein substrate, forming a phosphocysteinyl enzyme intermediate (Zhang, 1998; Zhang et al., 1994a; Zhang et al., 1998a; Wang et al., 2000). The catalytic cysteine has a pKa that is much lower than that of free cysteine and forms a thiolate anion. The apparent pKas of the nucleophilic cysteines in YopH, PTP1B, and VHR have been estimated as 4.7, 5.4, and 5.6 respectively (Zhang and Dixon, 1993; Lohse et al., 1997; Denu et al., 1995; Evans et al., 1996). The pKa of thiolate anion in LMW PTP from bovine heart (BPTP) was 4.0 (Wang et al., 2000). The thiolate ion of cysteine is stabilized by the conserved serine residue through hydrogen bonding (Zhang, 1998). The conserved arginine plays a role in binding the oxyanion of the phosphorylated substrate through its guanidinium group (Zhang, 1998; Zhang et al., 1994a; Zhang et al., 1998a). Arginine also stabilizes the
transition state of the phosphoenzyme intermediate (Zhang, 1998). The conserved aspartate functions as a general acid by donating the proton to the phenololate ion of the leaving group. In the second step, this same carboxylate side chain functions as a general base. It abstracts a proton from a water molecule, enhancing its nucleophilicity. The water molecule attacks the phosphoenzyme intermediate, releasing inorganic phosphate (Zhang, 1998; Denu et al., 1996; Wang et al., 2000). Figure 1-5 illustrates the two-step catalytic mechanism of a PTP.

Figure 1-5. A ping-pong mechanism for the reaction catalyzed by conventional PTPs, DSPs, and low molecular weight PTPs.
Biological Functions of PTPs:

PTPs can act as "on" or "off" switches for many cellular signaling pathways by catalyzing the removal of phosphoryl groups from tyrosine residues of protein substrates (Zhang, 1998; Denu et al., 1996; Wo et al., 2000). The dual specific protein phosphatase VHR from human, for example, down regulates c-Jun N-terminal kinase (JNK) in response to environmental stress and cytokines (Todd et al., 2002). Other examples of DSPs include the members of Cdc25 family. Cdc25A functions at the start of the cell cycle and regulates the G1/S transition (Zhang, 1998; Khaled et al., 2005). Cdc25B cooperates with Cdc25A to induce mitosis, but it also has a unique role in activating cyclin B1-Cdk1 at the centrosome (Lindqvist et al., 2005). Cdc25C not only plays a role at the G2/M transition but also in the modulation of DNA replication where its function is distinct from that of cdc25A (Turowski et al., 2005).

PTP1B, a member of cPTP superfamily, is a regulator of the insulin mediated signaling pathway. It dephosphorylates the tyrosine phosphorylated forms of the insulin receptor (IR) and insulin receptor substrate 1 (IRS-1) (Suryawan and Davis, 2002). Overexpression of PTP1B has been observed in insulin-resistant states associated with obesity. Inhibitors of PTP1B might provide tools for the treatment of type 2 diabetes and obesity by increasing insulin sensitivity (Rao et al., 2006).

It was determined that the members of LMW PTP superfamily are involved in Rho-mediated cytoskeletal rearrangements after stimulation by integrin and platelet-derived growth factor (Chiarugi, et al., 2000; Fiaschi et al., 2001).
Alzheimer brains, the low molecular weight phosphatase activity was significantly decreased compared to that in control brains, however, the high molecular weight and Zn\(^{2+}\) dependent acid phosphatase activity in control and Alzheimer brains was no different. These results suggest that reduced activity of the low molecular weight phosphatase might be linked to the aberrant protein tyrosine phosphorylation found in Alzheimer brains (Shimohama et al., 1993; Gong, et al., 1993; Lee et al., 2004)

**PTPs and Infectious Disease:**

It has been established that the pathogenic strategy of *Yersinia* includes the expression of an extracellular protein tyrosine phosphatase, YopH, which is essential for virulence. Various pathogenic strains of *Yersinia* are responsible for many human diseases ranging from gastrointestinal syndromes to the Bubonic Plague or Black Death (Zhang, 1988; Butler, 1985; Cozzone et al., 2004). The targets of YopH are eukaryotic host proteins such as p130cas (Black and Bliska, 1997), a docking protein that localizes to focal adhesions when phosphorylated on tyrosine residues in response to integrin engagement. Dephosphorylation of this protein by YopH causes cell detachment from the extracellular matrix (Bliska et al, 1993). SptP from *S. typhimurium* is also an essential virulence determinant, which targets eukaryotic host proteins and disrupts the actin cytoskeleton (Kaniga et al., 1996).
Protein Tyrosine Phosphorylation in *Bacteria*:

For a long time, protein phosphorylation on tyrosine has been considered a modification specific to eukaryotes (Kennelly and Potts, 1996). In these organisms, the reversible phosphorylation/dephosphorylation process, catalyzed by opposing reactions of protein tyrosine kinases and protein tyrosine phosphatases, is known to play a key role in a series of important biological functions including growth control, malignant transformation, and metabolism (Soulat et al., 2002). Evidences suggested that tyrosine phosphorylation is not exclusive to “higher” organisms. This cellular mechanism also occurs in bacteria and archaebacteria (Cozzone et al., 2004).

In bacteria, the first conventional PTP to be reported was YopH, which was encoded by a plasmid gene carried by pathogenic strains of *Yersinia pseudotuberculosis* (Guan and Dixon, 1990). Several other conventional PTPs, encoded by chromosomal DNA, have been characterized in bacteria including IphP from *Nostoc commune UTEX 584* (Potts et al., 1993), SptP from *Salmonella typhimurium* (Kaniga et al., 1996), and MptpB from *Mycobacterium tuberculosis* (Koul et al., 2000). PtpA from *Streptomyces coelicolor* was the first low molecular weight PTP reported in bacteria (Li and Strohl, 1996). Other members of low molecular weight PTP family in bacteria include Ptp from *Acinetobacter johnsonii* (Grangease, et al., 1998), Wzb from *E. coli* (Vincent et al., 1999), and MptpA from *M. tuberculosis* (Koul et al., 2000). On the other hand, evidence for presence of protein tyrosine kinase activity was first reported in *Acinetobacter calcoaceticus* (Daddsi an Cozzone, 1990).
In bacteria, the biological significance of protein phosphorylation on tyrosine is still unclear, because for a long time no individual protein kinase was detected and no endogenous protein substrates for protein tyrosine phosphatases were identified (Koul et al., 2000; Li and Strohl, 1996). Several attempts have been made to understand the possible biological role for tyrosine phosphorylation in bacteria. Approaches taken include analyzing changes in the patterns of tyrosine phosphorylation in response to variations in environmental conditions. It was observed that the tyrosine phosphorylation of two unidentified membrane proteins varies during the development of multicellular prokaryote Myxococcus xanthus, suggesting a role of phosphorylation during two important stages, aggregation and sporulation (Frasch and Dworkin 1996).

Evidence suggested that the pattern of tyrosine phosphorylation in different Streptomyces species (lividans, hygroscopicus, avendulae) varies with growth phase and that these patterns are influenced by culture conditions (Waters et al., 1994). Other studies revealed that in Streptomyces coelicolor A3(2) a dual specific kinase, AfsK, autophosphorylates on both serine and tyrosine residues. Disruption of the gene encoding the protein, afsK, had no effect on cell growth or differentiation, but did cause a significant decrease in blue pigment production (Matsumoto et al., 1994). Streptomyces coelicolor A3(2) also possesses a low molecular weight protein tyrosine phosphatase, PtpA. Studies of this phosphatase showed that this enzyme controls the production of secondary metabolites, namely A-factor, and the antibiotics actinorhodin and undecylprodigiosin (Umeyama et al., 1996).
Studies showed that a- and b- type flagellins of *Pseudomonas aeruginosa* are phosphorylated on tyrosine residues. This modification protects flagellins from endogenous proteases and serves as a cellular signal for appropriate export of flagellins (South et al., 1994). It was suggested that two low molecular weight protein tyrosine phosphatases, Wzb from *E. coli* and Ptp from *A. johnsonni*, are involved in synthesis or transport of exopolysaccharides as virulence factors (Grangeasse et al., 1998; Vinsent et al., 1999).

A light-dependent tyrosine kinase activity was observed in the cyanobacterium *Prochlorothrix hollandica*. Furthermore, an unidentified soluble 88-kDa protein was detected as a potential substrate for this protein kinase activity. The 88 kDa protein was phosphorylated when the cells were grown in high light conditions, but was not phosphorylated when cells were shifted to low light (Warner and Bullerjahn, 1994). Another cyanobacterium, *Nostoc commune* UTEX584, contains a dual specific phosphatase, called IphP (Howell et al., 1996; Potts et al., 1993). Several endogenous tyrosine phosphorylated proteins were detected from this organism (Potts et al., 1993). One of the tyrosine phosphorylated proteins is an uncharacterized 85-kDa protein. This protein was detected in cells grown on media supplemented with combined nitrogen, but not the nitrogen-deficient media that induces the formation of heterocysts (nitrogen fixing cells) (Potts et al., 1993). Protein tyrosine phosphatase and kinase activities also were reported from another cyanobacterium, *Anabaena* sp. strain PCC 7120. Cell extracts of this organism contained three tyrosine phosphorylated proteins of Mr ~27, 36, 52 kDa, detected using anti-
phosphotyrosine antibody under nitrogen fixing conditions (McCartney et al., 1997).

Synechocystis sp. PCC 6803:

My research is focused on protein tyrosine phosphorylation in the photosynthetic cyanobacterium Synechocystis sp. PCC 6803. The complete genomic sequence of this organism has been published (Kaneko et al., 1996), which facilitates the analysis of functional relationships among various protein kinases, protein phosphatases and phosphoproteins. Genomic sequence analysis suggests that this organism contains a tractably small set of signaling molecules. Synechocystis sp. PCC 6803 encodes approximately 30 potential protein Ser/Thr/Tyr kinases and protein Ser/Thr/Tyr phosphatases, and 40 histidine kinases (Zhang, et al., 1996; Kaneko et al., 1996; Marine et al., 2003). These analyses indicate that the phosphorylation network in this species is approximately 100-fold less quantitatively complex than that of a mammalian cell (Hanks and Hunter, 1995). The simplicity of this species provides a model for understanding more complex phosphorylation networks found in eukaryotes.

Synechocystis sp. PCC 6803 is a biologically sophisticated organism. It carries out a diverse range of metabolic activities and other biological functions, which enable it to grow in a wide range of environments (Zhang, 1996). This organism possesses refined sensor and regulatory systems. Further analysis of these systems will provide valuable information related to the molecular regulatory function that they possess (Stock, et al., 2000; Kennelly and Potts,
Synechocystis PCC 6803 possesses a high degree of genetic malleability (Kaneko et al., 1996; Zhang, 1996; Irmler, 2001), which enables the determination of physiological consequences of phosphorylation and the messages to which it responds.

### Protein Kinases and Phosphatases in Synechocystis sp. PCC 6803:

Eleven open reading frames encoding potential eukaryotic-like protein kinases were found by genome analysis of Synechocystis sp. PCC 6803 (Kaneko et al., 1996, Shi et al., 1998, Zhang et al., 1998). Seven of these potential protein kinases (SpkA, SpkB, SpkC, SpkD, SpkE, SpkF, SpkG) were characterized as the members of Pkn2 (protein kinase N2, a serine/threonine kinase or “Hanks-type” kinase) subfamily of the eukaryotic protein kinases (Kamei, et al, 2002). SpkA and SpkB are involved in cell motility, whereas the biological functions for SpkC to SpkG remain unclear (Kamei, et al, 2002).

Three open reading frames were identified that encode potential protein serine/threonine kinases of Spo/Rsb family. The protein product of one of ORFs, slr1861, was characterized as a protein serine/threonine kinase (Shi et al., 1999a). Genetic studies indicated that Slr1861 is involved in the regulation of carbon metabolism in Synechocystis sp. PCC 6803 (Beuf et al., 1994).

Genomic sequence analysis identified eight ORFs encoding potential serine/threonine phosphatases of the PPM superfamily. One example is slr1860, whose predicted protein product Slr1860, was identified as a member of PPM superfamily (Shi et al., 1999a). The gene, slr1860, was found in a cluster with the
Rsb/Spo-like protein kinase Slr1861, and two potential phosphoproteins, Slr1856 and Slr1859. This cluster of open reading frames is known as icfG gene cluster in *Synechocystis* sp. PCC 6803 (Beuf et al., 1994). Another example is the identification of *sll1771*, whose potential protein product was termed PphA (Irmler and Forchhammer, 2001). This enzyme is involved in dephosphorylation of serine phosphorylated PII signaling protein, which helps to maintain cellular nitrogen and carbon status. The predicted product of ORF *sll1033*, SynPPM3, was characterized from this organism as another member of the PPM superfamily (Li et al., 2005). This enzyme is able to dephosphorylate phosphoserine and as well as phosphotyrosine containing proteins *in vitro*.

The ORF *sll1387*, whose potential protein product (SynPPP1) has been identified as a member of PPP superfamily (Li et al., 2005). This phosphatase is able to dephosphorylate phosphoserine and phosphotyrosine containing proteins *in vitro* without addition of any exogenous metal ions cofactors, which is an unusual behavior for bacterial PPPs (Li et al., 2005).

No ORF encoding a conventional protein tyrosine phosphatase has been identified by genome analysis of *Synechocystis* sp. PCC 6803. However, three ORFs, encoding low molecular weight PTPs have been identified (Shi et al., 1998). One is *slr0946*, whose potential gene product was characterized as arsenate reductase (Li et al., 2003). The functional properties of the other two ORFs have not been reported.
Phosphoproteins in *Synechocystis* sp. PCC 6803:

PsbH: The gene product of *psbH* has been identified from thylakoid membranes of *Synechocystis* sp. PCC 6803 as a 6 kDa protein (Race and Gouranis, 1993). This protein became phosphorylated in both dark and light *in vitro* in presence of $[^{\gamma-32P}]-ATP$. Phosphorylation was inhibited by oxidizing conditions, and in presence of DCMU (dichlorophenyl dimethyl urea) or zinc ions. Cyanobacterial PsbH is homologous with proteins from higher plants and algae, even though it has a deletion of 27 nucleotides from 5'end of the coding sequence and is missing the phosphorylation site (threonine in position two) of the corresponding chloroplast product from higher plants (Michel and Bennett, 1987; Dedner et al., 1988). The predicted C-terminal sequence of PsbH is highly conserved among different species (Mayes and Barber, 1990). The phosphorylation site of cyanobacterial PsbH has not been identified, but it is suggested (Race and Gouranis, 1993) that it could be either the threonine at position five or the serine at position sixteen in this protein, which are conserved with higher plants and algae (Race and Gouranis, 1993). A number of proposals have been made for the possible role of phosphorylation in PsbH protein in plants and algae, which include i) regulation of excitation energy transfer (Allen, 1992), ii) protection from photo inhibition (Sundby, 1990; Sundby et al., 1990; Packham, 1988), iii) function as a redox sensor in the control of gene expression during photosynthesis (Allen, 1992), iv) stabilization of photosystem II and bicarbonate binding on its acceptor site (Komenda et al., 2002). It is suggested that an analog of PsbH protein in
cyanobacteria might be involved in same functions mentioned above (Race and Gouranis, 1993).

Slr1856 and Slr1859: The predicted gene products of slr1856 and slr1859 can be phosphorylated by the protein Ser/Thr kinase Slr1861 of Rsb/Spo family \textit{in vitro}. Site directed mutagenesis indicated that Slr1856 and Slr1859 were phosphorylated on Ser-54 and Ser-57, respectively. The phosphorylated proteins were substrates for Slr1860, a ser/thr phosphatase of the PPM superfamily, \textit{in vitro} (Shi et al., 1999a).

PII protein: It has been determined that a 13 kDa protein in \textit{Synechocystis} sp. PCC 6803, is highly homologous with seryl phosphorylated PII protein of the cyanobacterium \textit{Synechococcus} PCC 7942 (Forchhammer and Tandeau, 1994). This PII protein transmits signals of cellular carbon and nitrogen status through the phosphorylation. It was studied that a Ser/Thr phosphatase of PP2C superfamily in \textit{Synechocystis} sp. PCC 6803, termed PphA, is responsible for dephosphorylation of PII-phosphorylated protein (Irlmer and Forchhammer, 2001).

Phycobilisome linker proteins: It has been reported that the colorless linker proteins of phycobilisomes in \textit{Synechocystis} sp. PCC 6803 were phosphorylated on threonine residues (Piven et al., 2005). Immunological assay with phosphothreonine antisera indicated that two rod linkers proteins, $L_R^{33}$ and $L_R^{35}$;
a core membrane linker protein, L\textsubscript{CM}\textsuperscript{99}; and a rod core linker protein L\textsubscript{RC}\textsuperscript{27} are phosphothreonine containing proteins detected by immunoreactions (Piven et al., 2005). Superscripts indicate the apparent molecular size of the linker proteins in kDa. Phosphorylation of linker proteins might occur during assembly of phycobilisome hexamers, while dephosphorylation of these proteins may serve as signal for the protein degradation, which leads to disassembly of the phycobilisome complex in \textit{Synechocystis} sp. PCC 6803 (Piven et al., 2005; Anderson et al., 1998).

Phycocyanin: Phycocyanin is a major bilin-containing protein in the phycobilisome complex of \textit{Synechocystis} sp. PCC 6803 and functions as a light harvesting antenna for the transfer of the excitation energy to photosynthetic reaction centers (Allen, 1992; Grossman et al., 1993; MacColl, 1998). The question of whether phycocyanin is phosphorylated has been controversial. It has been reported (Mann and Newman, 1999) that \(\beta\)-phycocyanin in \textit{Synechocystis} sp. PCC 6803 was phosphorylated \textit{in vitro} using \([\gamma\textsuperscript{-32P}]\text{ATP}\). Site-directed mutagenesis revealed that Ser-50 of \(\beta\)-phycocyanin was the site of phosphorylation (Mann and Newman, 1999). Similar work has been done both \textit{in vivo} and \textit{in vitro} in the cyanobacterium \textit{Synechococcus} sp. PCC 6301 using \(32\text{P}\) orthophosphate or \([\gamma\textsuperscript{-32P}]\text{ATP}\) (Sanders et al., 1989; Harrison et al., 1991; Allen and Holmes, 1986; Sanders and Allen; 1987). It was repeatedly observed that major phosphorylated bands appeared at 18.5 kDa and 15 kDa on SDS-gels. It was suggested that these protein bands corresponded to polypeptides of the
phycobilisome complex (Sanders et al., 1986; Allen et al., 1985). The 18.5 kDa protein was proposed as β-phycocyanin (Sanders and Allen, 1987; Sanders et al., 1989). However, Piven and co-workers (Piven et al., 2005) could not detect any phosphorylation in β-phycocyanin using phosphoprotein staining gel dye Pro-Q Diamond or phospho-threonine antisera. Since phycocyanin autofluoresces, they suggested that the signals obtained for phycocyanin from Pro-Q staining arose due to its self-fluorescence.

**Significance of the Project:**

My research involves the study of protein tyrosine phosphorylation in cyanobacterium *Synechocystis* sp. PCC 6803. Genome analysis of this organism has revealed ORFs which encode potential protein tyrosine phosphatases. This study focused on characterizing the functional properties of the potential low molecular weight protein tyrosine phosphatase, Slr0328, in this organism. It was asked whether this hypothetical phosphatase was able to hydrolyze the phosphoesters from phosphoprotein substrates and if so whether this enzyme was specific to phosphotyrosyl proteins or possessed dual specificity. What phosphoproteins were targeted by this enzyme for its catalytic activity inside the cell? What was the biological function of this enzyme? Was it unique to this photosynthetic bacterial species or it was a common function to many other bacteria such as involvement in exopolysaccharide synthesis? This study developed one portion of a comprehensive effort to characterize the network of protein phosphorylation processes in this organism. This research also served as
a model for the more complex protein phosphorylation networks encountered in eukaryotic organisms including plants, animals, and humans.

In cyanobacteria, many protein serine/threonine kinases (Kamei et al., 2002), protein serine/threonine phosphatases (Shi et al., 1998a; Irmler and Forchhammer 2001; Li et al., 2005), dual specific protein phosphatases (Li et al., 2005; Howell et al., 1996), and endogenous serine/threonine phosphorylated proteins (Shi et al., 1998a; Race and Gouranis, 1993; Forchhammer and Tandeau, 1994; Piven et al., 2005) have been reported. But no protein tyrosine kinase, nor any member of conventional PTPs or low molecular weight PTPs have been described so far from the cyanobacteria. Many endogenous tyrosine phosphorylated proteins have been detected from different cyanobacterial species, but not identified (Warner and Bullerjahn, 1994; Potts et al., 1993; McCartney et al., 1997). In Cyanobase, genome sequences of eight different cyanobacterial strains have been published. Many ORFs whose predicted putative protein products are homologs with low molecular weight PTPs have been reported, but their functional properties have not been characterized so far (alr5068, Anabaena sp. PCC 7120; tlr1810, Thermosynechococcus elongatus BP-1; gll3174, Gloeobacter violaceus PCC7421; pmm1591, Prochlorococcus marinus MED4; pmt1688, Prochlorococcus marinus MIT9313; synw2026, synw0915, Synechococcus sp. WH8102). My research provided significant information of tyrosine phosphorylation in cyanobacterium Synechocystis sp. PCC 6803.
Specific Aims: Specific aims of this study are outlined as follows:

1. To identify potential protein tyrosine phosphatases from *Synechocystis* sp. PCC 6803.

To achieve specific aim-1 the following objectives were selected:

i) Clone and sequence *slr0328*.

ii) Express and purify the predicted product of *slr0328*.

iii) Determine whether recombinant Slr0328, named as SynPTP, has phosphatase activity.

iv) Compare kinetic parameters of SynPTP with those of known low molecular weight bacterial PTPs.

2. To determine whether SynPTP specifically hydrolyzes the phosphomonoesters from tyrosine residues as protein substrates or it is a dual specific phosphatase.

To achieve specific aim-2 the following objectives were selected:

i) Compare the catalytic activity of SynPTP toward α-naphthyl phosphate (α-NP) and β-napthyl phosphate (β-NP).

ii) Examine the catalytic activity of SynPTP toward exogenous protein substrates, which are phosphorylated on either serine or tyrosine residues.

iii) Determine the effects of known phosphatase inhibitors on the catalytic activity of the SynPTP.
3. To identify physiologically relevant enzyme-substrate relationships between SynPTP and phosphoproteins within *Synechocystis* sp. PCC 6803.

To achieve specific aim-3 the following objectives were selected:

i) Isolate potential substrates for SynPTP from *Synechocystis* sp. PCC 6803 through substrate trapping.

ii) Determine whether proteins isolated by substrate trapping contain phosphotyrosine.
CHAPTER II

Materials and Methods:

Instrumentations:

All chemicals were purchased from Sigma (St. Louis, Missouri) or Fisher (Pittsburgh, Pennsylvania), unless otherwise indicated. All radioisotopes were purchased from Perkin Elmer Life and Analytical Sciences (Boston, Massachusetts). PCR was performed using a GeneAmp PCR System Model 9600 (Applied Biosystem). Spectrophotometric measurements were performed using a Hitachi UV-2000 spectrophotometer. An Accumet Model AB15 pH Meter (Fisher Scientific) was used to measure the pH of all solutions. A Sorvall Superspeed RC2-B centrifuge equipped with an SS-34 or GSA rotor (Newton, Connecticut), or Fisher Scientific Marathon 12Kbr centrifuge (Pittsburgh, Pennsylvania) was used to pellet cells. A Model W185 sonifier cell disruptor, equipped with a microprobe from Heat Systems-Ultrasonics (Plainview, N.Y.), was used to lyse the cells. EC4000P Series 90 programmable power supply from E-C Apparatus Corporation (Holbrook, New York) was used for SDS-PAGE analysis. A Pharmacia Multidrive XL power supply, LKB Multi Temp II thermostatic circulator, and LKB Multiphor II were used for isoelectric focusing (Uppsala, Sweden). Western blotting was performed with a Bio-Rad Power Pac 200 and Electrophoretic Blotting unit (Hercules, CA). Liquid scintillation counting was performed using a Beckman (Fullerton, California) Coulter Model LS 6500 Multipurpose Scintillation Counter. Electronic scans of SDS-gels and
immunoblots were made on a flatbed UMAX Astra 1220S Scanner (Fremont, CA).

**Materials for Molecular Biology:**

The apparatus used for agarose gel electrophoresis was from Owl Scientific Inc. (Portsmouth, New Hampshire). *Pfu Turbo* DNA Polymerase for PCR was purchased from Stratagene (La Jolla, California). Oligonucleotides for PCR were synthesized by Life Technologies, (Rockville, Maryland). Minipreps for DNA isolation were performed using QIA prep Spin Miniprep Kit from Qiagen (Velencia, CA). *E. coli* strain TOP10 competent cells and BL21 star DE3 from Invitrogen (San Diego, California) were used for plasmid production and protein expression respectively. DNA sequencing was performed by the DNA Sequencing Facility, Virginia Bioinformatics Institute, (Blacksburg, Virginia). Miller LB Agar and Miller LB broth were purchased from Fisher (Pittsburgh, Pennsylvania).

**Methods:**

**Genomic DNA Isolation from *Synechocystis* sp. PCC 6803:**

The procedure was followed as described by Li et al., 2001. In brief, *Synechocystis* sp. strain PCC 6803 wild type (obtained from ATCC) was grown in 50 ml BG-11 media at 25°C with continuous shaking at 100 rpm under white light at an intensity of approximately 10 μmol photons m⁻² s⁻¹. Cells were grown until OD₇₃₀nm reached 1.0 (exponential), and then harvested by centrifugation at
2700xg for 20 min at 4°C. Pellets were washed 3 times with 15 ml sterile water. Pellets were resuspended in 500 μl sterile TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.5) and transferred to a sterile microcentrifuge tube. Cells were lysed with glass beads (Sigma, G-9143) using a bead beater (Biospec, Bartelsville, OK) for 30 sec at 4800 rpm and immediately kept on ice for 1 min. This cycle was repeated for 4 times. The mixture was centrifuged at 14000xg for 10 minutes. The supernatant liquid was collected and beads were discarded. Next, 50 μl of 10% SDS was added to the supernatant liquid, the mixture was agitated on a vortex mixture, incubated at 42°C for 5 minutes, and immediately placed on ice. One volume of phenol was added to the mixture, agitated on a vortex mixer, and centrifuged for 5 min at 14,000xg. The upper layer (aqueous layer) was collected in a sterile microcentrifuge tube and extracted with phenol twice or more in the same manner. Next, one volume of chloroform was added to the aqueous layer, the resulting mixture was agitated on a vortex mixer, and then centrifuged for 5 min at 14,000xg. Upper layer was collected and extracted twice or more with chloroform as described above and the aqueous layer was collected. Three volumes of chilled 100% (v/v) ethanol were added (1.5 ml ethanol:500 μl aqueous layer). The resulting mixture was then inverted slowly 4 times, and centrifuged at 14000xg for 10 min. The supernatant liquid was discarded. The DNA pellet was washed 3 times with cold 75% (v/v) ethanol resuspended in 100 μl of sterile water.
PCR (Polymerase Chain Reaction) for slr0328:

PCR amplification of slr0328 was carried out in a total volume of 50 µL that contained 550 ng genomic DNA from Synechocystis sp. PCC 6803 as template, 10 pmole of each forward and reverse primer (listed in table 1), 1 µL dNTP mix (Invitrogen, San Diego, California), 5 µL 10X Pfu turbo buffer, 2 µL Pfu Turbo DNA polymerase, according to the following program: incubation for 3 min at 95°C, followed by 30 cycles consisting of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds. For the last cycle, the incubation period at 72°C was extended to 5 minutes.

Table 2-1. A list of primers used to amplify ORF slr0328 and vectors used to clone slr0328.

<table>
<thead>
<tr>
<th>Vectors</th>
<th>Forward Primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET101/D-TOPO</td>
<td>5′-caccatgaaattgttattttgtaggtaac-3′</td>
<td>5′-attaaccaattccttgcccaagc-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′-ctattaaccaattccttgcccaagc-3′</td>
</tr>
<tr>
<td>pET100/D-TOPO</td>
<td>5′-caccatgaaattgttattttgtaggtaac-3′</td>
<td>5′-ctaattaaccaattccttgcccaagc-3′</td>
</tr>
</tbody>
</table>

Cloning and Transformation of PCR Products of slr0328:

PCR products were cloned into two different vectors as described below.

(i) Cloning of slr0328 into a vector encoding a C-terminal histidine tag.

Fresh PCR reaction mixture, 2~4 µL, was combined with 1 µL pET101/D TOPO vector (Invitrogen, San Diego, California), and 1 µL salt solution (1.2 M...
NaCl, 0.06 M MgCl$_2$). The vector encodes a C-terminal hexahistidine sequence and an epitope for anti-V5 antibody for detection of recombinant fusion proteins (Figure 2-1). The reaction mixture was brought to a final volume of 6 $\mu$L with sterile water and incubated for 5-30 minutes at room temperature. Next, 2 $\mu$L of the mixture was added to one vial of thawed One Shot chemically competent E.coli strain TOP10 cells. The resulting mixture was incubated on ice for 30 minutes. Following that, the reaction mixture was incubated for 30 seconds at 42$^\circ$C and immediately placed on ice. Approximately 250 $\mu$L SOC media (containing 2%(w/v) Tryptone, 0.5% (w/v) Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl$_2$, 10 mM MgSO$_4$, and 20 mM glucose, and sterilizing by autoclave), pre-warmed to 37$^\circ$C, was added to the reaction mixture. The tubes were capped tightly and the reaction mixture was agitated at 200 rpm for 30 minutes at 37$^\circ$C. Following that, a portion of the mixture, 25-50 $\mu$L, was spread onto pre-warmed LB-Ampicillin (100 $\mu$g/ml) plates. The inoculated plates were incubated overnight at 37$^\circ$C. Approximately 10 colonies were picked and used to inoculate 3 ml portions of LB media containing ampicillin (100$\mu$g/ml). The mixture was incubated overnight at 37$^\circ$C with shaking. Plasmids were isolated from overnight culture using QiA prep Spin Miniprep Kit (Velencia, CA).
Figure 2-1. A diagram of pET 101/D-TOPO vector. Panel A shows the features of the vector encoding a V5 epitope and a hexa-histidine tag. Panel B displays a portion of the sequence of the vector. Blue arrowheads indicate the position at which the PCR product of *slr0328* was inserted.
To express the predicted gene product of *slr0328* with no fusion tag, the following approach was taken. ORF *slr0328* was cloned into the same pET 101/D-TOPO vector as described in the previous section with the exception that, the reverse primer for PCR contained a stop codon (underlined, listed in Table 2-1). The addition of a stop codon ahead of the sequence encoding the fusion tag of the vector prevents the translation of hexahistidine sequence and the recognition epitope for the fusion protein (Figure 2-1).

(ii) Cloning of *slr0328* into a Vector which Encodes an N-terminal Histidine tag.

The PCR product for *slr0328* was cloned into pET100/D-TOPO vector (Invitrogen, San Diego, California) in a similar manner as described in section (i). The vector encodes the sequences for an N-terminal hexahistidine tag, an enterokinase cleavage site, and an epitope for anti-Xpress antibody to detect the recombinant fusion proteins (Figure 2-2). The primers used for the PCR were listed in Table 2-1.
Figure 2-2. A Diagram of pET100/D-TOPO vector. Panel A shows a schematic diagram of the vector, which encodes a hexahistidine tag, an epitope for anti-Xpress antibody, and an enterokinase cleavage site. Panel B shows a portion of the sequence of the vector. Arrowheads in blue indicate the site of ligation of the PCR product.
DNA Sequencing:

DNA sequencing of purified plasmids was performed at Virginia Bioinformatics Institute (VBI) DNA Sequencing Facility (Blacksburg, Virginia). The gene, slr0328, 487 bp, encoding a full length protein tyrosine phosphatase, was sequenced in the forward direction using T7 promoter primer.

Site–Directed Mutagenesis:

To mutagenically alter the catalytic cysteine, Cys7, to serine of the predicted gene product of slr0328, the following mutagenic primer was used: 5'-ATGAAATTGTTATTTGTTAGTTAGGTAACATTTG-3' (the codon for serine is underlined). Site-directed mutagenesis of slr0328, cloned into the pET101/D TOPO vector (encoding a C-terminal hexahistidine sequence), was performed using Promega’s GeneEditor™ in vitro site-directed mutagenesis system (Madison, Wisconsin) according to the manufacturer’s protocol. Figure 2-3 details the procedure for mutagenic alteration of slr0328. In brief, a plasmid with slr0328 cloned into the pET101/D TOPO vector was used as dsDNA template. Alkaline denaturation of dsDNA, 5’ phosphorylation of mutagenic oligonucleotides (primers), and mutagenesis reactions were performed according to the procedures provided by Promega. Following that, the mutagenesis reaction mixture was heated to 75°C for 5 minutes and then cooled slowly to 37°C. Mutant strands were synthesized with T4 DNA polymerase and T4 DNA ligase and transformed into BMH71-18 mutS competent cells. Cells were grown overnight with the GeneEditor™ antibiotic selection mix. Plasmid DNA was isolated from
the overnight culture and transformed into JM 109 or TOP10 competent cells. Transformants were selected on LB/Ampicillin plates with GeneEditor™ antibiotic selection mix. The insertion of the point mutation was confirmed by sequencing the mutant plasmids.

Figure 2-3. A schematic diagram of GeneEditor™ *in vitro* mutagenesis procedure (Promega, Madison, Wisconsin).
Following mutagenic alteration of catalytic Cys$_7$ to Ser, mutagnic alteration of Asp$_{125}$ to Ala was performed using a QuickChange II Site-Directed Mutagenesis Kit (Stratagene,CA) according to manufacturer’s protocol (Figure 2-4). The primers used for the mutagenesis reactions were 5’-CGGGAAGTTCCCGCCCCCTATTATGGC-3’ as the forward primer and 5’-GCCATAATAGGGGCGGGGAACTTCCCG-3’ as the reverse primer. In brief, DNA amplification was performed using *Pfu Ultra* DNA polymerase (Stratagene, CA). The reaction mixture for PCR contained 50ng of plasmid encoding the mutagenically altered Cys$_7$ to Ser slr0328 (from previous section) as template, 10pmole of each mutagenic oligonucleotide (forward and reverse primer), 1µL dNTP mix, 5µL of 10X reaction buffer, 1µL *Pfu Ultra* DNA polymerase in a total volume of 50µL. PCR was carried out using the following program: 30 seconds incubation at 95°C, 15 cycles consisting of 95°C for 30 sec, 55°C for 60 sec, and 68°C for 6 min and 30 sec. Following temperature cycling, the reaction mixture was placed on ice for 2 min to cool the mixture to 37°C. Once cooled, 1µL of *Dpn I* restriction enzyme (10U/µL, Stratagene) was added and the mixture incubated for 1 hr at 37°C to digest parental dsDNA. Next, 1µL of the *Dpn I* treated reaction mixture was used to transform 50 µL of XL1-Blue Supercompetent cells (Stratagene). Transformants were selected from LB/ampicillin plates. The insertion of the point mutation was confirmed by sequencing the mutant plasmids.
Figure 2-4. An overview of the QuickChange II site directed mutagenesis method (Stratagene, CA).

**Solutions for Molecular Biology:**

The solutions for Luria Broth (LB) media, LB plates, TE pH 8.0, stock solutions for antibiotics (ampicillin, chloramphenicol, kanamycin), or stock solutions for IPTG were prepared according to Sambrook et. al., (1989).
Agarose Gel Electrophoresis:

Samples of genomic DNA, plasmid DNA, or PCR products were routinely analysed using agarose gel electrophoresis as described in Sambrook et al., (1989). High melt agarose gels, 1.0% (w/v) were prepared using 1xTAE buffer (Tris-Acetate-EDTA, consisted of 0.04M Tris base, pH 8.0, 1.15%(v/v) acetic acid, and 1 mM EDTA) containing 0.4% (w/v) ethidium bromide. DNA samples were prepared by mixing one volume of 5x loading buffer (Bio Rad) with four volumes of sample. Electrophoresis was conducted at 100 volts in TAE buffer.

Expression of the Predicted Gene Product of slr0328 in *E. coli*:

The predicted gene product of *slr0328* was named SynPTP and will be referred to as such for the rest of the thesis. Protein expression was performed according to manufacturer’s protocol, Invitrogen (San Diego, California). In brief, following plasmid purification, 1µL (5-10ng) of the purified plasmid encoding recombinant SynPTP was added to one vial of competent cells of *E. coli* BL21 star (DE3) One Shot Cells and incubated on ice for 30 minutes. The mixture was then heated for 30 seconds at 42°C, without shaking, and immediately placed on ice. Next, 250µL of SOC medium was added. Tubes were capped tightly and incubated at 37°C for 30 minutes with shaking at 225 rpm. The entire transformation mixture was added to 3ml LB media containing ampicillin (100µg/ml). Cells were incubated overnight at 37°C with shaking at 225rpm. The overnight culture was transferred to a 1L flask containing 200mL LB media with 100 µg/mL of ampicillin, and shaken at 225 rpm, at 37°C, until A_{600nm} was in the
range of 0.5 to 0.9. Isopropyl-β-thiogalactopyranoside (IPTG) was then added to a final concentration of 1.0 mM and the cultures were incubated for 5 hrs at 25°C with shaking. The cells were harvested by centrifugation at 10,000xg for 30 minutes at 4°C, and stored at –80°C until needed.

**Purification of Recombinant SynPTPs Containing a “histidine tag”:**

The cell pellet from a 200mL culture was thawed and resuspended in 5 mL of 50 mM Tris HCl (pH 7.5) containing 50mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.1 mg/mL of lysozyme, and 5% (v/v) glycerol. The resulting solution was incubated on ice for 30 min. The cells were lysed by sonic disruption with four bursts of 20 s each at 50 watts of a Heat Systems-Ultrasonics Inc, Model W185 Sonifier Cell Disruptor equipped with a microprobe. The resulting lysate was centrifuged for 20 min at 10,000xg and 4°C. The supernatant was filtered through a 0.45 µm filter to remove any particulate materials and applied to a metal ion affinity column for purification of the recombinant protein following established protocols (Shi et al., 1999a). In brief, a 1 ml bed volume of column was prepared using Chelating Sepharose Fast Flow (Pharmacia Biotech, Uppsala, Sweden). The column was washed with 10 bed volumes of sterile water. Next, the column was charged with Ni²⁺ by applying 3 bed volumes of 200mM NiSO₄, followed by 10 bed volumes of sterile water. Equilibration of the column was performed with 10ml buffer A, which contains 50 mM Tris HCl, pH 7.5, 50mM NaCl, and 5% (v/v) glycerol. The supernatant, prepared from the cell lysate as described above, was passed through the column twice. The column
then was washed with 2x10 bed volumes of buffer containing 10 mM imidazole. Bound proteins were eluted in a step gradient with same buffer in which imidazole concentration was increased from 10mM to 250 mM. Eluted fractions were collected in 1 ml volume and analyzed for the presence of recombinant SynPTPs by SDS-PAGE in 12% (w/v) Acrylamide gels.

**Purification of Recombinant SynPTP Lacking a Fusion Tag:**

The cell pellet from a 200 mL culture was thawed, resuspended in 5 mL of 10 mM Tris HCl (pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride and 0.1 mg/ml of lysozyme, and incubated on ice for 30 min. The cells were lysed by sonic disruption as described above and the resulting lysate was centrifuged for 20 min at 10,000xg at a temperature of 4°C. The supernatant was collected and filtered through a 0.45 µm filter to remove any particulate materials present. Next, the filtered supernatant was applied to a strong anion exchange column of Q Sepharose Fast Flow (Amersham Pharmacia Biotech, Uppsala, Sweden), 1-ml bed volume, that had been equilibrated with 10 mM Tris HCl, pH 7.5, and 5% (v/v glycerol). The column was washed with the same buffer containing 10 mM NaCl and adherent proteins eluted with a salt gradient of 25 to 500 mM NaCl in 10 mM Tris HCl, pH 7.5, containing 5%(v/v) glycerol. Fractions, 1 ml, were collected and analyzed by SDS-PAGE.
Protein Assay:

Protein concentration was determined by the method of Bradford (Bradford, 1976) using Coomassie Protein Assay reagent from Bio-Rad (Hercules, CA). A standard curve was generated using 0-20 \( \mu \text{g} \) bovine serum albumin in deionized water. For unknowns, the quantity of sample was adjusted such that the resulting absorbance at 595 nm fell in the linear range of the standard curve.

SDS-PAGE (Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis):

SDS polyacrylamide resolving gels, 12% or 15% (w/v), stacking gels, 5% (w/v), and running buffer (25 mM Tris, pH 8.3, 192 mM glycine, 0.1% (w/v) SDS) were prepared as described by Laemmli (Laemmli, 1970). Protein samples were prepared by mixing three volumes of sample with one volume of 4X loading buffer (200 mM Tris, pH 8.0, 400 mM DTT, 8% (w/v) SDS, 40% (w/v) glycerol and 0.4 % (w/v) bromophenol blue) (Sambrook et. al., 1989). The mixture was heated for 5 min at 100°C, quickly centrifuged to collect all liquid at the bottom of the tube, and then loaded onto the gels. Low range molecular weight markers, Precision Plus Protein Standards Dual Color (Bio-Rad, Hercules, CA), were loaded into one well of the gel to estimate the apparent molecular size of unknown proteins. Gels were run at a constant current of \(~25\text{mAmp/gel}\) until all prestained markers entered the resolving gel. The current was then increased to \(~50\text{ mAmp/gel}\) until the dye front reached within ½~1 inch of bottom of the gel. Gels were immersed in gel fixing solution (40%(v/v) methanol, 7%(v/v) acetic
acid) for 10 min. Gels were stained by soaking in 100 ml of dye, consisting of 50%(v/v) methanol, 10%(v/v) acetic acid, and 0.25%(w/v) Coomassie Brilliant R-250, for one hour. The gels were destained by soaking in several changes of destaining solution, consisting of (20%(v/v) methanol, 10%(v/v) acetic acid). Finally, the gels were wrapped with saran wrap and scanned by UMAX Astra 1220S Scanner (Fremont, CA).

**Western Blotting of Proteins onto PVDF (PolyVinylidene DiFluoride) Membranes:**

Following SDS-PAGE, proteins were transferred from polyacrylamide gels to an Immobilon-P (Millipore, MA) PVDF membrane using a wet transfer electroblotting system (Bio-Rad, Hercules, CA). The PVDF membrane was wetted with 100% methanol for 30 seconds, rinsed with water for five minutes, and soaked in blotting buffer (10 mM Caps, pH 11, 10% (v/v) methanol) for 10 minutes. The polyacrylamide gel containing the proteins was also soaked with blotting buffer. The assembly of the transblotting sandwich and transfer of proteins were performed according to the manufacturer's protocol (Bio-Rad, CA). Generally, proteins were electroblotted at 50 volts (constant volts) overnight at 4°C. After transfer of proteins to PVDF membrane, the membrane was treated for immunodetection with antibody to confirm the presence of recombinant proteins.
Immunodetection of Recombinant SynPTP Expressed with Fusion Tag:

Recombinant SynPTPs expressed with pET 101/D-TOPO vector (containing a V5 epitope) or pET 100/D-TOPO vector (containing an Xpress epitope) were detected immunologically using either anti-V5 antibody or anti-Xpress antibody. Following the electrophoretic transfer of proteins to the PVDF membrane, the membrane was soaked in 100% methanol for 10 seconds, and then the membrane was placed on a benchtop at room temperature for at least 4 hours to complete dry. Following that, the membrane was incubated at room temperature for 1 hour with either anti-V5 antibody or anti-Xpress antibody at the dilution of 1:5000 (v/v) (2 µl: 10,000 µl) in 10 ml TBST (10 mM Tris base, 150 mM NaCl, 0.05% (v/v) Tween-20, pH 7.5) with 1% BSA. Next, the membrane was washed with 2x10ml TBST for 10 seconds, each time. Following that, the membrane was incubated with mouse anti-goat alkaline phosphatase conjugated secondary antibody at the dilution of 1: 2500 (v/v) (4 µl: 10,000 µl) in 10 ml TBST with BSA, for 1 hour, at room temperature. After that, the membrane was washed twice with 10ml TBST for 10 seconds, each time. Finally, the membrane was immersed in 10 ml of BCIP (BromoChloroIndolyl Phosphate, Sigma) solution (1 tablet dissolved in 10 ml water) for 1~5 minutes to visualize the recombinant protein as a dark purple band. The membrane was washed with water to stop color development. The membrane was dried, scanned, and stored in a dark place.
Immunodetection by Anti-Phosphotyrosine Antibody:

After blotting, the PVDF membrane was incubated with TBST buffer containing 1%(w/v) BSA (bovine serum albumin) for 1 hour at room temperature. Next, the membrane was incubated with rabbit monoclonal antibody RC20 (B. D. Transduction Laboratory, Rockville, MD) at a dilution of 1:50,000 in 25 ml TBST buffer with 1% BSA for 1 hour at room temperature. The antibody is phosphotyrosine specific and conjugated with horseradish peroxidase. Following incubation, membrane was washed six times with 50 ml of TBST, each for 30 min. An enhanced chemiluminescence system (Pierce, Rockford III) was used according to the manufacturer’s protocol for the detection of immunoreactive proteins.

2D Gel Electrophoresis:

All materials and equipment were from Pharmacia (Amersham Biosciences, Uppsala, Sweden). Isoelectric focusing (IEF) of protein samples was conducted according to manufacturer’s protocol. In brief, proteins were precipitated by combining one volume of sample with three volumes of ice-cold acetone and incubating overnight at −20°C (approximately 18 hr). The precipitated protein was collected by centrifugation at 16000xg for 15 min in a microcentrifuge. Residual acetone was evaporated by air-drying. Following evaporation, 125 µL (for a 7 cm IPG strip) of rehydration solution (9 M urea, 4% (w/v) CHAPS detergent, 100 mM DTT, trace amounts of bromophenol blue, and 5 µL appropriate IPG (Immobiline pH gradient) buffer), was added to the protein
sample. The mixture was allowed to sit at room temperature for at least 2 hrs. The sample was then applied to the Dry Strip Reswelling Tray and overlaid with an IPG dry strip (7 cm pH 4-7 linear or pH 3-10 linear). The strip was allowed to rehydrate for at least 16 hrs at room temperature. IPG cover fluid was used to cover the IPG strip. The following day, the strip was rinsed with deionized water and placed on the aligner of the dry strip tray, with the acidic end (pointed end or positive end) facing towards the red anode. Each electrode was aligned across the strip and IPG cover fluid was used to cover the strip during isoelectric focusing. The Multiphor II system (Pharmacia) with a cooling plate connected to a thermostatic circulator set at 20°C was used to perform isoelectric focusing. The following programs were used for IEF. For a 7 cm strip, pH 4-7 linear: phase 1, 200V, 2mA, 5W, 1 minute, 1Vh; Phase 2, 3500V (Voltage will increase slowly from 200V to 3500V, mA will gradually decrease from 2mA to 0.2mA), 5W, 1:30hrs, 2800Vh; Phase 3, 3500V (voltage will stay constant), 2mA, 5W, 1:30hrs, 5200Vh. Total duration of program was 3 hours and 1 minute, 8001 Vh. For 7cm strip, pH 3-10 linear, the Pharmacia Multidrive XL power supply was programmed as follows: phase 1, 200V, 2mA, 5W, 1 minute, 1Vh; Phase 2, 3500V (Voltage will increase slowly from 200V to 3500V, mA will gradually decrease from 2mA to 0.2mA), 5W, 1:30hrs, 2800Vh; Phase 3, 3500V, 2mA, 5W, 1:05 hrs, 3700Vh; total duration of program was 3hrs 35mins for 6500Vh. After IEF, the IPG strip was used immediately for the second dimension, SDS-PAGE, or stored in a 15mL Falcon tube at –80°C until needed.
Prior to the second dimension, the IPG strip was incubated with SDS equilibration buffer (50mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and a trace amount of bromophenol blue) in two steps. First, the strip was immersed in 5 mL equilibration buffer containing 50 mg DTT for 15 minutes on a shaker at room temperature. Next, the strip was immersed in 5 mL equilibration buffer containing 125 mg iodoacetamide and shaken at room temperature for 15 minutes. Finally, the strip was applied to the top of the SDS-polyacrylamide separating gel (12% (w/v)) and sealed with 0.5% (w/v) agarose containing a trace amount of bromophenol blue. Electrophoresis was performed as described for SDS-PAGE procedure.

Expression and Purification of GST/lyn Kinase:

_E. coli_ containing a pGEX-KT plasmid encoding a GST/lyn kinase fusion protein was a generous gift from Marietta Harrison and Harry Charbonneau from Purdue University. GST/lyn kinase was expressed and purified as described in Howell et. al., (1996). In brief, an aliquot of glycerol permanent stock of the bacterial cells (_E. coli_ strain Top10 competent cells) was streaked onto a LB/ampicillin plate, which was incubated overnight at 37°C. A single colony was picked from the plate and used to inoculate 5 mL of LB/ampicillin media. The mixture was incubated overnight at 37°C and used to inoculate 1 liter of LB/ampicillin media. The mixture was incubated at 37°C with continuous shaking until OD_{600nm} reached 0.7. IPTG was added to a final concentration of 0.2 mM and cells cultured for 3 hrs at 37°C, then harvested by centrifugation at 10,000xg
for 30 minutes at 4°C. Cell pellets were stored at –80°C until needed. Cell pellets were resuspended in 10 mL PBS (phosphate buffered saline) containing 2 mM EDTA, 0.1% (v/v) β-mercaptoethanol, 2 mM PMSF (phenylmethylsulfonyl fluoride), and protease inhibitor cocktail (Sigma). Cells were lysed by sonication using a Heat Systems-Ultrasonics, Inc; Model W185 Sonifier Cell Disruptor equipped with a microprobe. After sonication, Triton X-100 was added in a final concentration of 1% (w/v) and the mixture was incubated on ice for 15 minutes. Cell debris and other insoluble materials were removed by centrifugation at 10,000xg for 20 minutes at 4°C. The supernatant liquid was collected and mixed with 1 mL of a 50% (v/v) suspension of glutathione-agarose beads in PBS. The slurry was incubated overnight at 4°C with gentle rotation. This step was performed in a 15 mL centrifuge tube (Falcon). The glutathione-agarose beads were pelleted in a table top centrifuge and the supernatant liquid was removed. The agarose beads were washed at least 4 times with 10 mL ice cold PBS containing 2 mM EDTA, 0.1% (v/v) β-mercaptoethanol, 2 mM PMSF, and protease inhibitor cocktail (Sigma). The beads were suspended as a 50% (v/v) slurry in PBS containing 10% (v/v) glycerol and stored at –20°C. To dissociate adherent proteins from the beads, reduced glutathione at a final concentration of 10 mM was added to a portion of GST/lyn kinase agarose beads. The mixture was incubated at room temperature for 30 minutes with rotation and beads were removed by centrifugation. Supernatant was analyzed for protein concentration by Bradford protein assay.
Preparation of $^{32}$P-Phosphotyrosyl RCM-Lysozyme:

Lysozyme was reduced, carboxymethylated and maleylated (RCML) as described by Tonks et. al., (1988). RCML was phosphorylated on tyrosine residues as follows: A 1 ml reaction mixture contained 50 mM Tris, pH 7.5, 2 mM DTT, 10mM MgCl$_2$, 0.015% (w/v) Brij 35, 50 mM ATP, 500 $\mu$ci of [$\gamma$-$^{32}$P] ATP, 1 mg RCML, and 500 $\mu$l GST/lyn kinase bound to glutathione-agarose beads in an Eppendorf tube. The reaction mixture was incubated approximately 18 hrs at room temperature with constant agitation on a tube rotator. Following incubation, the agarose beads were removed by centrifugation in a microcentrifuge at 16000xg for 5 min. The supernatant liquid containing phosphotyrosyl RCML was collected and separated from unreacted [$\gamma$-$^{32}$P] ATP by precipitation with 20%(w/v) cold TCA as described by Tonks et al., (1988).

Preparation of $^{32}$P-Phosphotyrosyl Casein:

Casein was phosphorylated on tyrosine residues under identical reaction conditions as described for RCM-Lysozyme ($^{32}$P-Tyr), except casein (1mg, Sigma) was used instead of RCM-lysozyme. After removal of GST/lyn kinase-agarose beads by centrifugation, phosphotyrosyl-casein was purified from the reaction mixture by gel filtration chromatography. The supernatant was applied to a 2x18cm column of Sephadex G-25. The column was equilibrated with 50mM Tris, pH 7.5, containing 1mM DTT and 0.1 mM EGTA (Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetra acetic acid). Fractions, 1 ml, were collected. Portions, 10$\mu$l, of each
fraction were added to 1 ml scintillation fluid to measure radioactivity. Fractions containing $^{32}$P-phosphotyrosyl casein were pooled and stored at –20°C.

**Preparation of $^{32}$P-Phosphoseryl Casein:**

Phosphorylation of casein on serine residues was performed using the catalytic subunit of cAMP dependent protein kinase. The reaction was carried out overnight at room temperature in a 1 ml volume containing 50 mM Tris, pH 7.0, 1 mM DTT, 10 mM MgCl$_2$, 0.1 mM EGTA, 1 mg casein, 10 µg PKA catalytic subunit (Sigma, St. Louis), and 10 µCi [$\gamma$-$^{32}$P] ATP. The lyophilized protein kinase was resuspended in 100 µl of 50 mM Tris, pH 7.0, 1 mM DTT and allowed to stand at room temperature for 15 min prior to addition of the reaction mixture. The radiolabeled phosphoprotein was purified by gel filtration chromatography using Sephadex G-25 as described for the preparation of phosphotyrosyl casein.

**Preparation of $^{32}$P-Phosphoseryl RCM-Lysozyme:**

$^{32}$P-phosphoseryl RCM-Lysozyme was prepared under identical conditions as described for $^{32}$P-phosphoseryl casein, except that RCM, 1 mg, was used instead of casein. $^{32}$P-phosphoseryl RCM-Lysozyme was purified by TCA precipitation as described for $^{32}$P-phosphotyrosyl RCM-Lysozyme.

**Preparation of $^{32}$P-Phosphotyrosyl Myelin Basic Protein (MBP):**

MBP ($^{32}$P-Tyr) was prepared under the same reaction conditions as described in the preparation of $^{32}$P-phosphotyrosyl casein, except that MBP (Sigma, St.
Louis), 1 mg, was substituted for casein. The radiolabeled protein was purified using gel filtration chromatography as described in the previous section.

**Preparation of \(^{32}\text{P}-\text{Phosphoseryl Myelin Basic Protein (MBP):}\)**

MBP (\(^{32}\text{P}-\text{Ser}\)) was prepared under the same reaction conditions as described in the preparation of \(^{32}\text{P}-\text{phosphoseryl casein}, with the exception of MBP, 1mg, was substituted for casein. The radiolabeled phosphoprotein was purified in the similar manner as described for the preparation of \(^{32}\text{P}-\text{phosphoseryl casein.}\)

**Protein Phosphatase Assay:**

Phosphatase activity toward \(^{32}\text{P}-\text{tyrosyl phosphorylated or} \(^{32}\text{P}-\text{seryl phosphorylated substrates was measured by the following procedure. SynPTP, 0.1 \text{~2.0 µg, was incubated at 30°C for 20 min in a volume of 40 µL containing 100 mM MES, pH 6.5, 2 mM DTT, 1 mM EDTA, 0.5 mg/ml bovine serum albumin (BSA), and 4 µM protein bound [}^{32}\text{P}] \text{ phosphate. At the end of the incubation period, the reaction was stopped by addition of 200 µL of chilled 20% (w/v) trichloroacetic acid. Following vigorous agitation on a vortex mixer, the assay solution was centrifuged for 10 min at 14,000xg. A 40 µL sample of the supernatant liquid was removed and dispersed into 1 mL of scintillation fluid, and the liberated }^{32}\text{P}, was measured in a scintillation counter.}**
Phosphohydrolase Activity toward pNPP:

Protein phosphatase assay toward pNPP was performed by the following method. SynPTP, 0.1-1.0 µg, was incubated at 30°C for 30 min in a volume of 200µL. The reaction mixture contained 100 mM MES, pH 6.5, 2 mM DTT, 1 mM EDTA, and 10 mM pNPP. The reaction was terminated by the addition of 700 µL of 0.5 M NaOH, and the absorbance of the resulting solution was measured at 410 nm. The amount of para nitrophenolate released was quantified by using a molar extinction coefficient of 18,300 M⁻¹ cm⁻¹.

Malachite Green Assay:

Phosphohydrolase activity of SynPTP toward organophosphoesters was measured as described by Lanzetta, et. al., (1979). SynPTP was used in the amount of 200 ng in a volume of 200 µL, containing 100 mM MES, pH 6.5, 2 mM DTT, 1 mM EDTA, and 10 mM organophosphate. The mixture was incubated at 30°C for 30 min. Following the incubation, a 50 µL aliquot of the reaction mixture was removed and assayed for the inorganic phosphate by addition of 800 µL Malachite Green reagent (color reagent), followed by 100 µL 34% (w/v) of sodium citrate solution. After incubating at room temperature for 15 min, the absorbance of the resulting solution was measured at 660 nm. The amount of free phosphate was determined from a standard curve using K₂HPO₄ as standard.

Color reagent (Malachite Green reagent) was prepared on the day of assay by mixing one volume of 4.2% (w/v) ammonium molybdate prepared in 4N
HCl with three volumes of 0.045% (w/v) Malachite Green. Color reagent was allowed to incubate at room temperature for 15 min prior to use.

**Growth of *Synechocystis* sp. PCC 6803:**

BG11 (ATCC 616) media contained 1.5g NaNO₃, 0.04 g K₂HPO₄, 0.0375g MgSO₄ (anhydrous), 0.036g CaCl₂·2H₂O, 0.006g citric acid, 0.006g ferric ammonium citrate, 0.001g sodium EDTA, 0.054g Na₂CO₃·10H₂O, 1mL trace metal mix (2.86g H₃BO₃, 1.81g MnCl₂·4H₂O, 0.22g ZnSO₄·7H₂O, 0.39g Na₂MoO₄·2H₂O, 0.079g CuSO₄·5H₂O, 49.4mg Co(NO₃)₂·6H₂O per liter in deionized water) in one liter deionized water. The pH of the media was adjusted to 7.1 with 1N HCl or 1N NaOH and sterilized by autoclaving.

**Cell Growth:**

*Synechocystis* sp. PCC 6803 wild type strain (obtained from ATCC) was grown in 250 ml BG-11 media at 25°C with continuous shaking at 100 rpm under white light at an intensity of approximately 10 μmol photons m⁻² s⁻¹. Cells were grown until OD₇₃₀nm reached to 1.0 (exponential), then harvested by centrifugation at 2700xg for 20 min at 4°C, and stored at –20°C until needed.

**Preparation of Whole Cell Extracts of *Synechocystis* sp. PCC 6803:**

The cell pellet from a 250 ml culture was thawed and resuspended in 15 ml cell lysis buffer (50 mM MES, pH 6.5, 1mM DTT, 1mM EDTA, 0.1mM sodium fluoride, 10mM NaCl, and protease inhibitor cocktail). Suspended cells were
lysed by 3 passes through a chilled French pressure cell at 100 MPa. Cell debris were removed by centrifugation at 5000xg for 10 min. Extracts were stored at 4°C for further analysis.

**Preparation of Soluble Fractions of *Synechocystis* sp.PCC 6803:**

The whole cell extracts of *Synechocystis* sp. PCC 6803 were centrifuged at 100,000xg, using a Beckman 50Ti rotor, for two hours at 4°C. Supernatant liquid was saved as the soluble fraction. The pellet was collected as the membrane fraction and stored at –20°C for further analysis.

**Extraction of Proteins from Membrane Fractions of *Synechocystis* sp.PCC 6803:**

Chlorophyll content was determined from 20 ml of whole cell extracts of *Synechocystis* sp. PCC 6803 using an extinction coefficient of 74,000 M⁻¹cm⁻¹ at 679 nm (Kruip et al., 1993). The membranes were incubated with Triton-X-100 (w/w chlorophyll: detergent ratio of 1:10) in 10 ml buffer containing 50mM MES (pH 6.5), 10 mM NaCl, 1 mM DTT, 1 mM EDTA, and protease inhibitor cocktail (Sigma) at 4°C for 3 hours. Following that membrane fractions were centrifuged at 14,000xg for 10 min (Armbrust et al., 1996). Supernatant (green color), containing membrane proteins, was collected and kept at 4°C for further analysis.
Coupling of SynPTP or Mutagenically Altered SynPTP to NHS (N-Hydroxy Succinimide)-Activated Sepharose:

NHS (N-hydroxysuccinimide) forms chemically stable amide linkages with primary amino groups. Hi-trap NHS-activated Sepharose (Amersham Bioscience, Uppsala, Sweden) was used to immobilize 5 mg purified active SynPTP or purified mutagenically altered SynPTP according to the manufacturer’s protocol (Amersham Biosciences, Uppsala, Sweden). In brief, NHS activated Sepharose, 2 ml bed volume, was placed in a 15 ml Falcon tube. Sepharose beads were sedimented by centrifugation at 500xg for 5 minutes. Supernatant liquid was discarded and washed with 5 bed volumes of deionized water by gentle rotation in a tube rotator for 5 minutes, followed by centrifugation at 500xg for 5 minutes. Next, beads were washed with 5 bed volumes of 1mM cold HCl by gentle agitation in a tube rotator for 5 minutes. Purified recombinant SynPTPs were dialyzed in buffer containing 50mM MES, pH 6.5, 1mM DTT, 1mM EDTA, and protease inhibitor cocktail (Sigma, St. Louis). Dialyzed enzymes in the amount of 5 mg, in 10 ml volume, were added to the HCl washed beads and gently agitated in a tube rotator for 3 hours at room temperature. Beads were sedimented by centrifugation at 500xg for 5 minutes and supernatant liquid was saved for further analysis. A small portion of the Sepharose beads and a small volume of supernatant (~250 µl), from both recombinant SynPTP bound Sepharose or mutagenically altered SynPTP bound Sepharose, were examined for phosphohydrolase activity of using pNPP (para-nitrophenyl phosphate) to verify that the coupling reaction was completed. It was observed that the solution of
Analysis of Soluble Fractions:

The soluble fractions containing 15 mg protein in 10 ml supernatant liquid was applied to the Sepharose beads (active SynPTP or mutagenically altered SynPTP bound Sepharose beads) mentioned in previous section and incubated
at 30°C for overnight. Following day, beads were centrifuged at 500xg for 5 minutes. Supernatant was collected and kept at 4°C for further analysis. The beads were washed with five bed volumes of buffer containing 50 mM MES (pH 6.5), 10 mM NaCl, 1 mM DTT, 1 mM EDTA, and protease inhibitor cocktail for 10 minutes by gentle agitation in a tube rotator. Bound proteins were eluted in a step gradient with 50 mM NaCl to 250 mM NaCl (50 mM, 100 mM, 150 mM, 200 mM, 250 mM) in the same buffer. Each eluted fraction was collected in a 2 ml volume. Sepharose beads were further washed with 250 mM NaCl or 1M NaCl in the same buffer containing 50 mM phosphotyrosine and collected in 2 ml volume each. Eluted fractions were analyzed on SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody.

Purification of Phycocyanin from Soluble Fractions of *Synechocystis* sp. PCC 6803 Cell Extracts by Anion Exchange Chromatography:

DE-52 Sepharose (Sigma), 2 ml, was placed in a 15 ml Falcon tube. The resin was washed with 5 volumes of deionized water, by gently agitation for 5 min in a tube rotator, followed by centrifugation at 500xg for 5 min. Supernatant was discarded and resin was equilibrated twice with 10 volumes of buffer A (50 mM MES, pH 6.5, 1 mM DTT, 1 mM EDTA, 0.1 mM sodium orthovanadate, 0.1 mM sodium fluoride, and protease inhibitor cocktail (Sigma)) with gentle rotation in a tube rotator twice, 10 min each. Resin was sedimented by centrifugation at 500xg for 5 min and supernatant was discarded. The soluble fractions of *Synechocystis* sp. PCC 6803 cell extracts were prepared as described in a
previous section. The soluble fractions, containing approximately 50 mg of total protein, were added to the tube and incubated with gentle agitation for 2 hrs at room temperature in a tube rotator. Next, the mixture was centrifuged for 5 min at 500xg. The supernatant liquid was collected and kept at 4°C for analysis by SDS-PAGE. The resin was washed twice with 10 ml of buffer A containing 10 mM NaCl by agitation in a tube rotator twice for 15 min. The bound proteins were eluted in a step gradient with buffer A containing salt concentration from 25 mM to 250 mM NaCl (25 mM, 50 mM, 75 mM, 100 mM, 125 mM, 150 mM, 175 mM, 200 mM). Eluted fractions were collected twice in 2.5ml volume, analyzed by SDS-PAGE, and immunodetected with anti-phosphotyrosine antibody.

Purification of Phycocyanin by Size Exclusion Chromatography:

A 2x65 cm column of Sephadex G-75 (Sigma) was equilibrated with buffer A containing 50 mM MES, pH 6.5, 1 mM DTT, and 1mM EDTA). The supernatant liquid from 125mM, 150mM and 175mM NaCl steps of DE-52 resin were mixed together and the mixture containing 5 mg of total protein in 10 ml volume, was loaded to the Sphadex G-75 column. The column was developed with buffer A including 5% glycerol, 0.1 mM sodium othovanadate, 0.1 mM sodium fluoride, protease inhibitor cocktail, and 1 mM PMSF. Blue-colored eluates from the column were collected in 1 ml fraction. The ratio of $A_{620}/A_{280}$ was measured for each fraction. Those fractions, which had an $A_{620}/A_{280}$ ratio in the range of 3.8 to 4.2, were saved (Moreno et al., 1997; Minkova et al., 2003). A portion of these
fractions was analyzed on SDS-PAGE and Western blotting using an anti-phosphotyrosine antibody.

**Mass Spectral Analysis:**

**Extraction of Tryptic Digested Peptides from SDS-Gel:**

The procedure, described in Lower et al., (2003) was followed for tryptic digestion of polypeptides and extraction of digested peptides. In brief, the section of the Coomassie stained SDS-gel containing the band/spot of interest was excised using a clean razor blade, sliced into small pieces approximately 1x1mm² and placed into a 0.5 ml siliconized tubes (VWR). At the same time a gel piece from protein free region of the SDS-gel was excised as a control for identifying trypsin autoproteolysis products. To the gel pieces, 100 µl or sufficient volume of 25 mM ammonium bicarbonate containing 50% acetonitrile (v/v) was added to cover the gel particles, and the mixture was agitated for 35~45 min on a vortex mixer. Gel loading pipette tips were used to remove and discard the pale blue colored free liquid from the tube. This step was repeated for 5 times. After removing all the Coomassie stain, the gel pieces were dehydrated by adding 100 µl acetonitrile. At this point gel pieces were shrunken and became opaque –white in color. Acetonitrile was removed from the tube and gel slices were then dried in a speed vacuum centrifuge for 10 minutes.

Reduction and alkylation of cysteine residues of proteins within the gel matrix were performed as follows: sufficient volume of 10 mM DTT in 25 mM ammonium bicarbonate, ~ 30 µl, was added to cover the gel pieces, and the
mixture was incubated at 55°C for 1 hr. Next, the DTT solution was replaced with roughly the same volume of 55 mM iodoacetamide in 25 mM ammonium bicarbonate and incubated for 45 min at room temperature in the dark. Next, the iodoacetamide solution was removed and gel particles were washed with ~100 µl of 25 mM ammonium bicarbonate, pH 8, for 10 min with occasional mixing on a vortex mixer. The wash solution was removed and 100 µl of 25 mM ammonium bicarbonate containing 50%(v/v) acetonitrile was added. The mixture was continuously agitated for 10 min on a vortex mixer. The supernatant liquid was removed and gel pieces were dehydrated by adding ~100 µl of acetonitrile. Acetonitrile was removed from the tube and gel pieces were dried in a vacuum centrifuge for 10 min.

Next, the dried gel pieces were rehydrated by adding 1 volume of 25 mM ammonium bicarbonate, pH 8, containing 0.1mg/ml sequencing grade trypsin (Promega). The mixture was agitated for 5 min using a vortex mixer and incubated overnight at 37°C. Next, 2 volumes of deionized water were added and the mixture was agitated for 5 min. The supernatant liquid was removed and transferred to a new siliconized tube. Two additional extractions were performed by adding 2 volumes of 5%(v/v) TFA (trifluoroacetic acid) containing 50% acetonitrile and agitated for 5 min as before. Each time the supernatant was removed and pooled together with the previous extractions. The pooled supernatants, containing the extracted tryptic peptides, were concentrated to a final volume of ~10 µl in a vacuum centrifuge.
Next, the tryptic peptides were eluted from the digested mixture by using OMIX C18 tips (Varian Inc, CA) as follows: the OMIX C18 tip was wet by pipetting 50% acetonitrile and dispensing to waste. This step was repeated 3 times with fresh solution. The tip was equilibrated by pipetting 0.2% TFA in 2% acetonitrile and dispensing to waste by repeating 3 times with fresh solution each time. Next, the tryptic peptides were bound to the tips by pipetting a 20 µl aliquot from the digested mixture and dispensing to a new siliconized tube. This binding step of tryptic peptides by pipetting and dispensing of digest solutions was performed for 10 cycles to get better recovery of the peptides. Following that, the tip was washed by pipetting 0.2% TFA in 2% acetonitrile and dispensing to waste. This step was repeated 5 times with fresh solution each time. The tip was further washed by pipetting 5% formic acid in 2% methanol and dispensing to waste. This step was repeated 5 times with fresh solution each time. Finally, bound peptides were eluted by pipetting 20 µl 5% formic acid in 95% methanol and dispensing to a siliconized tube. The pipetting/dispensing step was repeated 5 times. Liquid was spun down and dried by airflow to near dryness. The pellet was resuspended in ~ 5µl of 5% formic acid in 50% methanol. Finally, the sample was ready for mass spectral analysis. Mass spectrometric analysis was conducted using a nano-electrospray source from Proxeon (Odense, Denmark) attached to a Finnigan TSQ Quantum Ultra AM mass spectrometer (Thermo Electron Corp. West Palm Beach, FL).
In-solution Trypsin Digestion and Extraction of Tryptic Peptides:

Protein samples were concentrated by precipitation. Chilled methanol was added to the sample in the ratio of 4:1 (v/v). The mixture was incubated on ice for at least 1 hr, then centrifuged at 14,000xg for 30 min at 4°C. The pellet was resuspended in 200 µl of freshly prepared 100 mM ammonium bicarbonate. To enhance the solubilization of protein pellets, 100% acetonitrile was added to a final concentration of 5% (v/v) to the mixture.

Following that, sequencing grade trypsin (Promega) was added to the mixture in 1:25 (w/w) ratio to total protein (1 µg trypsin : 25 µg protein). The mixture was incubated overnight at 37°C. Next, 5% (v/v) TFA stock solution was added to the mixture to bring the final concentration of TFA to 0.2% (v/v) and the pH of the solution was adjusted to ≤ 3 by adding 98% formic acid.

Next, the tryptic peptides were eluted by using OMIX C18 tips (Varian Inc, CA) and analyzed by mass spectral analysis as described in previous section.
Chapter III

Results:

Previous Studies:

Search for Protein Tyrosine Phosphatases in *Synechocystis* sp. PCC 6803:

A homology search for protein tyrosine phosphatases (PTPs) in *Synechocystis* sp. PCC 6803 was previously performed in our laboratory (Shi et al., 1998). Three open reading frames (ORF), *slr0328*, *slr0946*, and *slr1617*, were identified whose predicted gene products shared the active site sequence L/VC-X$_5$-RS/T, and conserved aspartate of low molecular weight (LMW) protein tyrosine phosphatases (Grangreasse et al., 1998; Li and Strohl, 1996; Soulat et al., 2002; Vincent et al., 1999). The potential protein product of *slr0946* was characterized as an arsenate reductase (Li et al., 2003). The functional properties of ORF *slr1617* have not been described.

Open reading frame *slr0328* was cloned and its protein product was expressed with an N-terminal histidine tag in *E. coli* by Songhai Lin, a previous student in our laboratory. The continued study of this enzyme served as my research project. Figure 3-1 illustrates the conserved signature sequence for the LMW PTPs, the amino acid sequence for Slr0328, also named SynPTP, and a comparison of the sequence of SynPTP with other known LMW PTPs.
Figure 3-1. Slr0328 (SynPTP) possesses the active site sequence and conserved aspartate similar to known LMW PTPs. (A) The conserved sequence for LMW PTP. (B) The amino acid sequence of SynPTP. The active site sequence VC-X5-RS/T (red) is present near the extreme N-terminus of the catalytic domain, and conserved aspartate is located at 110 residues to the C-terminal side of conserved serine followed by a proline. (C) The alignment of the deduced amino acid sequence of SynPTP with other LMW PTPs, constructed by using the CLUSTALW program of Biology Workbench. The conserved sequences are shown in red. Shown are the DNA-derived amino acid sequences of LMW PTPs from Homo sapiens, GenBank accession no. P24666; S. cerevisiae, GenBank accession no. P40347; M. tuberculosis H37Rv, GenBank accession no. CA94656; S. coelicolor A3 (2), GenBank accession no. P53433; Synechocystis sp. PCC 6803 (Slr0328), GenBank accession no. Q55335; E. coli (Wzb), GenBank accession no. P77153; A. johnsonii, GenBank accession no. CA75430.
Specific Aim-1: To characterize a potential protein tyrosine phosphatase, Slr0328, from Synechocystis sp. PCC 6803.

Goals of specific aim-1:

i) Clone and sequence slr0328

ii) Express and purify the predicted product of slr0328, SynPTP

iii) Determine whether recombinant SynPTP possesses phosphatase activity

iv) Compare kinetic parameters of SynPTP with those of other known low molecular weight PTPs

i) Cloning and Sequencing of slr0328:

ORF slr0328 was cloned into various vectors as described in Materials and Methods.

ii) Expression and Purification of Predicted Protein Product of slr0328:

Expression and purification of protein product slr0328, named SynPTP was described in Materials and Methods. The purity of recombinant SynPTPs was assessed by SDS-PAGE (Figure 3-2).

Analysis of Purified Fractions of Recombinant SynPTP Expressed with a C-terminal His Tag or an N-terminal His Tag:

It was observed that the apparent molecular mass of the recombinant SynPTP or mutagenically altered Cys7Ser SynPTP, expressed with C-terminal His tag (20 kDa), matched the calculated molecular mass (21 kDa) of those
fusion proteins (Figure 3-2A). The presence of fusion proteins was confirmed by Western blot analysis using Anti-V5 antibody (Figure 3-2B). The apparent molecular weight of recombinant protein SynPTP expressed with an N-terminal His tag was also found to be consistent with the calculated mass of this fusion protein. The identity of this fusion protein was verified by immunoreaction with Anti-Xpress antibody (Figure 3-2C,D).

**Analysis of Purified Fractions of Recombinant SynPTP without a Fusion Tag:**

The major band from cells expressing SynPTP having no fusion tag was found to be at the expected size on polyacrylamide gel, 18 kDa (Figure 3-2E, Lane 2). The presence of this major band was not observed when the cells were grown in the absence of IPTG, which indicated that recombinant SynPTP was not expressed under this condition (Figure 3-2E, Lane 3).
Figure 3-2. Analysis of the purified recombinant SynPTP by SDS-PAGE.

(A) Recombinant SynPTP expressed with a C-terminal His tag was analyzed by SDS-PAGE. Each lane contained equal amount of protein (3 µg). Lane 1: Protein markers; Lane 2: A soluble fraction of cell lysate of SynPTP expressed with a C-terminal His tag after induction with 1 mM IPTG; Lane 3: A soluble fraction of cell lysate without induction as a negative control; Lane 4: A purified fraction of the recombinant SynPTP with a C-terminal His tag after elution from metal column; Lane 5: A purified fraction of mutagenically altered C7S SynPTP with a C-terminal His tag. Gels were stained with Coomassie Blue. (B) Shown is the corresponding result of Western blot analysis of a duplicate gel from SDS-PAGE. Anti-V5 antibody was used to detect the epitope of fusion proteins. (C) Analysis of the recombinant SynPTP with an N-terminal His tag by SDS-PAGE. Lane 1 contained 3 µg of a purified fraction of the recombinant SynPTP. (D) Western blot analysis of the duplicate gel from SDS-PAGE. Anti-Xpress antibody was used to detect the epitope of the fusion proteins. (E) Analysis of the purified SynPTP having no fusion tag by SDS-PAGE. The protein, bound to the anion exchange column, was eluted by 100 mM NaCl containing 10 mM of Tris HCl pH 7.5, 5% (v/v) glycerol, and subjected to 12%(w/v) SDS gel. Each lane contained 3 µg of protein. Lane 1: Protein markers; Lane 2: A fraction of the purified protein, eluted by 100 mM NaCl and cells were grown in presence of 1 mM IPTG; Lane 3: A fraction, which was eluted by 100mM NaCl and cells were grown in the absence of IPTG.
iii) Examination of the Phosphatase Activity of Recombinant SynPTPs.

The protein phosphatase activity of recombinant SynPTPs was analyzed toward Casein ($^{32}$P-Tyr) and RCML ($^{32}$P-Tyr) as protein substrates. Para-nitrophenyl phosphate (pNPP), a common substrate for PTPs, was also used to examine the phosphohydrolase activity of recombinant SynPTPs. Results are summarized in Table 3-1. It was observed that recombinant SynPTP expressed with a C-terminal His tag exhibited a similar level of phosphatase activity to the recombinant protein expressed without a fusion tag. However, the catalytic activity of the fusion protein with an N-terminal His tag was well below that of the C-terminally tagged protein or protein lacking a tag. Mutagenically altered C7S recombinant SynPTP also lacked detectable phosphatase activity.
Table 3-1. Comparison of phosphatase activity among recombinant SynPTPs.

<table>
<thead>
<tr>
<th>Recombinant proteins</th>
<th>Specific activity</th>
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<tbody>
<tr>
<td></td>
<td>RCML (³²P-Tyr)</td>
</tr>
<tr>
<td></td>
<td>(nmol/min/mg)</td>
</tr>
<tr>
<td>C terminal His tag</td>
<td>0.81 ± 0.01</td>
</tr>
<tr>
<td>N terminal His tag</td>
<td>0.048 ± 0.001</td>
</tr>
<tr>
<td>Without fusion tag</td>
<td>Not examined</td>
</tr>
<tr>
<td>mutagenically altered C7S</td>
<td>ND*</td>
</tr>
</tbody>
</table>

The phosphatase activity of various forms of SynPTP, 500 ng each, was measured using 4 µM casein (³²P-Tyr) or 4 µM RCML (³²P-Tyr) or 10 mM pNPP as described in Materials and Methods. Shown are the averages of triplicate determinations plus or minus standard error. *ND represents not detectable, less than 2% of the phosphatase activity of C-terminally tagged recombinant protein or protein lacking a tag.

These results indicate that recombinant SynPTPs possessed phosphatase activity. Since the recombinant SynPTP with a C-terminal His tag exhibited similar phosphatase activity to recombinant SynPTP having no fusion tag, either one of these proteins could be utilized for further experiments. Recombinant SynPTP with a C-terminal His tag was selected since it is simple to purify by metal ion column chromatography. No further experiments were conducted on recombinant SynPTP expressed with the N-terminal His tag due to its low specific activity.
iii) **Comparison of Kinetic Parameters of SynPTP with Other Known LMW PTPs toward pNPP as a Substrate.**

First, assay conditions for hydrolytic activity of SynPTP toward pNPP were optimized with respect to time, pH, temperature, and enzyme concentration (Figure 3-3). Kinetic analysis of the hydrolysis of pNPP by SynPTP was performed (Figure 3-4) and the parameters generated were compared with those of other known LMW PTPs. Results suggest that $K_m$ and $V_{max}$ values for SynPTP are comparable to those for other bacterial low molecular weight PTPs (Table 3-2). However, it was observed that $V_{max}$ value for SynPTP was much lower than the corresponding values for Stp1 from *S. pombe* (~4 fold) and BHPTP from bovine heart (~30 fold).
Figure 3-3. Influence of time, pH, temperature, and SynPTP concentration on catalytic efficiency of SynPTP toward pNPP. The Phosphohydrolase activity of SynPTP toward pNPP was determined as described in Materials and Methods, using 200 ng of purified recombinant enzyme at 30°C for 30 min, unless otherwise indicated. (A) Time dependence on catalytic efficiency of SynPTP for dephosphorylation of pNPP. (B) pH optima for dephosphorylation of substrate by SynPTP. The buffers used were MES at pH 5.0 to 7.0 adjusting with 1 M NaOH, and Tris Base at pH 7.5 and 8.0 adjusting with 1 M HCl. (C) Temperature dependence of SynPTP for dephosphorylation of the substrate. Enzyme was pre-incubated for 15 min at the individual temperature prior to the assay. Control indicates without any incubation prior to assay. Enzyme was kept on ice. (D) Hydrolysis of phosphoesters of pNPP by SynPTP as a function of SynPTP concentration. Amount of enzyme used was 0.1 µg to 1.0 µg. Shown are the results of triplicate determinations plus or minus standard error.
Figure 3-4. Kinetic analysis of SynPTP using pNPP as a substrate. Phosphatase activity of SynPTP was measured as described in Materials and Methods for pNPP. Shown is a Hanes-Woolf Plot describing the dependence of reaction rate on substrate concentration. Each point represents the triplicate determinations of plus or minus standard error.
Table 3-2. Comparison of kinetic constants of SynPTP with those of known LMW PTPs.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Enzyme</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (µmol/min/mg)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptomyces coelicolor A3(2)</td>
<td>PtpA</td>
<td>0.75</td>
<td>4.8</td>
<td>Li and Strohl, 1996</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>PtpB</td>
<td>1.5</td>
<td>1.4</td>
<td>Soulat et al., 2002</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>PtpA</td>
<td>1.2</td>
<td>33.6</td>
<td>Soulat et al., 2002</td>
</tr>
<tr>
<td>Acinetobacter Johnsonii</td>
<td>PTP</td>
<td>5.0</td>
<td>9.7</td>
<td>Grangreasse et al., 1998</td>
</tr>
<tr>
<td>E. coli</td>
<td>Wzb</td>
<td>1.0</td>
<td>4.6</td>
<td>Vincent et al., 1999</td>
</tr>
<tr>
<td><strong>Synechocystis sp. PCC 6803</strong></td>
<td>SynPTP</td>
<td>0.6</td>
<td>3.2</td>
<td><strong>This report</strong></td>
</tr>
<tr>
<td>Schizosaccharomyces pombe</td>
<td>Stp1</td>
<td>0.21</td>
<td>12.8</td>
<td>Zhang et al., 1995</td>
</tr>
<tr>
<td>Schizosaccharomyces cerevisiae</td>
<td>Ltp1</td>
<td>0.02</td>
<td>3.2</td>
<td>Ostanin et al., 1995</td>
</tr>
<tr>
<td>Bovine heart phosphatase</td>
<td>BHPTP</td>
<td>3.5</td>
<td>103</td>
<td>Wo et al., 1992</td>
</tr>
</tbody>
</table>

**Summary of Specific Aim-1:** A homology search for protein tyrosine phosphatases in *Synechocystis sp. PCC 6803* revealed that the predicted protein product of *slr0328*, named SynPTP, contains the signature motif (CX$_5$RS/T-X$_{85-110}$DP) similar to known low molecular weight protein tyrosine phosphatases. Preliminary results indicate that recombinant SynPTP catalyzes dephosphorylation of pNPP and tyrosine phosphorylated protein substrates (Casein (\(^{32}\)P-Tyr) or RCML (\(^{32}\)P-Tyr)). From kinetic analysis it was observed that $K_m$ and $V_{max}$ values for SynPTP toward pNPP are comparable to other known bacterial low molecular weight PTPs. In addition, it was observed that when the catalytic cysteine (C7) was altered to serine, recombinant SynPTP was no longer able to dephosphorylate the substrates mentioned above.
Specific Aim-2: To determine whether SynPTP specifically hydrolyzes the phosphomonoesters from tyrosine residues of protein substrates, or it functions as a dual specific phosphatase (DSP).

Goals:

i) Compare the catalytic activity of SynPTP toward $\alpha$-napthyl phosphate ($\alpha$-NP) and $\beta$-napthyl phosphate ($\beta$-NP).

ii) Examine the catalytic activity of SynPTP toward exogenous protein substrates that are phosphorylated on either serine or tyrosine residues.

iii) Determine the effects of potential phosphatase inhibitors on the catalytic activity of the SynPTP.

i) Compare the Catalytic Activity of SynPTP toward $\alpha$-Napthyl Phosphate and $\beta$-Napthyl Phosphate:

To examine the substrate specificity of SynPTP, several low molecular weight organophosphate compounds including isomers of napthyl phosphate were used. Relative enzymatic activity toward these substrates is summarized in Table 3-3. The napthyl phosphate isomers (Figure 3-5) were of particular interest, since they represent potential diagnostic substrates for differentiating PTPs from DSPs \textit{in vitro} (Zhang et al., 1994; Zhang, 1995; Howell et al., 1996, Savle et al., 2000).
The wide and shallow pockets of DSPs (Denu et al., 1995; Fauman et al., 1996) readily accommodate a variety of substrates including both isomers of napthyl phosphate. The deep and narrow binding pockets of PTPs (Ramponi et al., 1997) are able to accommodate $\beta$-napthyl phosphate because it can insert lengthwise to bring the phosphate ester in close proximity to the catalytic cysteine. However, the sideways approach required for the $\alpha$ isomer, cannot be accommodated by a narrow binding pocket of a PTP (Figure 3-6) (Chen et al., 1996; Zhang, 1995).
Figure 3-6. A schematic diagram of the substrate binding pockets of PTPs and DSPs. (A) The wide and shallow binding pocket of a DSP can accommodate both the napthyl phosphate isomers for the hydrolysis of phosphoesters by the nucleophilic attack of catalytic cysteine to the phosphates. (B) The deep and narrow binding pocket of a PTP allows entry of the beta isomer of the napthyl phosphate. However, the alpha isomer of napthyl phosphate cannot fit into the deep binding pocket of the PTP for dephosphorylation.
Table 3-3. Hydrolysis of low molecular weight organophosphate substrates by SynPTP.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Specific activity (µmol/min/mg)</th>
<th>Relative activity (%pNPP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-nitrophenyl-P</td>
<td>$3.2 \pm 0.26$</td>
<td>100</td>
</tr>
<tr>
<td>α-naphtyl-P</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td>β-naphtyl-P</td>
<td>$3.6 \pm 0.42$</td>
<td>117</td>
</tr>
<tr>
<td>Glucose-6-P</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td>1, 4, 5 Inositol phosphate</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td>Phosphotyrosine</td>
<td>$3.3 \pm 0.41$</td>
<td>104</td>
</tr>
<tr>
<td>Phosphoserine</td>
<td>ND*</td>
<td>ND*</td>
</tr>
</tbody>
</table>

Phosphohydrolase activity of SynPTP toward the listed organophosphoesters was measured as described in Materials and Methods using Malachite Green assay. The amount of enzyme used in the reaction mixture was 500 ng. All organophosphates were present at the final concentration of 10 mM in the reaction mixture. The enzyme activity is reported relative to that observed with pNPP, $3.2 \, \mu\text{mol/min/mg}$, which was set equal to 100%. ND* indicates not detectable, less than 2% or $0.07 \, \mu\text{mol/min/mg}$. All values are averages of triplicate determinations plus or minus standard error.
Table 3-4. Comparison of kinetic parameters for dephosphorylation of selected substrates by SynPTP and yeast LMW PTP (LTP1).

<table>
<thead>
<tr>
<th>Substrates</th>
<th>SynPTP</th>
<th>LTP1</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (mM)</td>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>$K_m$ (mM)</td>
</tr>
<tr>
<td>pNPP</td>
<td>0.6</td>
<td>1.18</td>
<td>0.017</td>
</tr>
<tr>
<td>β-Napthyl Phosphate</td>
<td>0.5</td>
<td>1.26</td>
<td>0.58</td>
</tr>
</tbody>
</table>

Figure 3-7. Determination of kinetic constants for the catalytic activity of SynPTP toward pNPP and β-Napthyl phosphate. Phosphatase activity was measured as described in Materials and Methods for pNPP and β-napthyl phosphate. Shown are the Hanes-Woolf Plots describing the dependence of the rate of reaction of SynPTP on pNPP ($\Delta$), and β-Napthyl phosphate ($\diamond$).
ii) **Examine the Catalytic Activity of SynPTP toward Exogenous Protein Substrates, which are Phosphorylated on either Serine or Tyrosine Residues.**

Several protein substrates including casein, myelin basic protein (MBP), and reduced carboxymethylated maleylated lysozyme (RCML), were used to examine the phosphoamino acid side chain specificity for SynPTP. These protein substrates were phosphorylated either on tyrosine residues or serine residues using $[^{32}\text{P}]\text{ATP}$ and either lyn kinase or the cAMP dependent protein kinase, respectively, as described in Materials and Methods.

The conditions for the assay of phosphatase activity were optimized with respect to enzyme concentration, time, pH, and temperature using RCML(32P-Tyr) as protein substrate (Figure 3-8).
Figure 3-8. Characterization of SynPTP for protein phosphatase activity with respect to time, enzyme concentration, pH, and temperature. Enzyme activity was assayed using 500 ng purified enzyme and 2 µM RCML (³²P-Tyr) as a substrate, at 30 °C for 20 min unless otherwise indicated. Protein phosphatase activity was measured as described in Materials and Methods. (A) Release of (³²P) phosphate from the substrate by SynPTP as a function of time. (B) Influence of pH on the catalytic efficiency of SynPTP for dephosphorylation of RCML (³²P-Tyr). The buffers used were MES at pH 5.0 to 7.0, Tris Base at pH 7.5 and 8.0 and pH of buffers were adjusted using either 1 M NaOH or HCl. (C) Effect of temperature on SynPTP for dephosphorylation of substrate. Enzyme was pre-incubated for 15 min at the individual temperature prior to the assay. Control represents no incubation prior to the assay. Enzyme was kept in ice prior to the activity assay. (D) Release of (³²P) phosphate from the substrate by SynPTP as a function of SynPTP concentration using from 0.1 µg to 2.0 µg of the enzyme. Shown are the results of triplicate determinations plus or minus standard error.
Table 3-5. Activity of SynPTP toward protein substrates.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Specific activity (pmol/min/mg)</th>
<th>relative activity (% RCML $^{32}$P-Tyr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCML ($^{32}$P-Tyr)</td>
<td>800 ± 16</td>
<td>100</td>
</tr>
<tr>
<td>Casein ($^{32}$P-Tyr)</td>
<td>1217 ± 60</td>
<td>162</td>
</tr>
<tr>
<td>MBP ($^{32}$P-Tyr)</td>
<td>283 ± 6</td>
<td>38</td>
</tr>
<tr>
<td>RCML ($^{32}$P-Ser)</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td>Casein ($^{32}$P-Ser)</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td>MBP ($^{32}$P-Ser)</td>
<td>ND*</td>
<td>ND*</td>
</tr>
</tbody>
</table>

The phosphatase activity of SynPTP, 500 ng, was measured toward the $^{32}$P labeled phosphoproteins listed above as described in Materials and Methods. All substrates were present at a final concentration of 4 µM protein bound $[^{32}$P] phosphate. Enzyme activity is reported in terms of both specific activity (pmol/min/mg) and relative to that observed with RCML ($^{32}$P-Tyr), which was set equal to 100%. ND* represents not detectable, less than 2%, or 16 pmol/min/mg. Shown are the results of triplicate determination plus or minus standard error.
Kinetic parameters for dephosphorylation of selected substrates by SynPTP.

Kinetic constants $K_m$ and $V_{max}$ for the enzyme were determined (Figure 3-9) toward $^{32}$P labeled phosphotyrosyl casein and RCML following the standard procedure described in Materials and Methods. Results are summarized in Table 3-6.

![Hanes-Woolf Plots](image)

Figure 3-9. Determination of kinetic constants for the catalytic activity of SynPTP toward casein ($^{32}$P-Tyr) and RCML ($^{32}$P-Tyr). The phosphatase activity of SynPTP was measured as described in Materials and Methods, with the exception that the concentration of each substrate was varied as shown. Shown are the Hanes-Woolf Plots describing the dependence of reaction rate of SynPTP on casein($^{32}$P-Tyr) ($\Delta$), and RCML($^{32}$P-Tyr) (□). Each point represents the average of triplicate determinations plus or minus standard error.
Table 3-6. Kinetic parameters of SynPTP toward casein (\(^{32}\text{P-Tyr}\)) and RCML (\(^{32}\text{P-Tyr}\)).

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Km ((\mu\text{M}))</th>
<th>(V_{\text{max}}) (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCML-(^{32}\text{P-Tyr})</td>
<td>3.5</td>
<td>1.3</td>
</tr>
<tr>
<td>Casein-(^{32}\text{P-Tyr})</td>
<td>2.0</td>
<td>1.5</td>
</tr>
</tbody>
</table>

iii) Determine the Effects of Potential Phosphatase Inhibitors on the Catalytic Activity of the SynPTP:

The catalytic activity of SynPTP was further examined by analyzing the effect of various compounds known to be inhibitors of protein tyrosine phosphatases. The objective of this experiment was to determine whether the phosphatase activity of SynPTP displayed a similar pattern of sensitivity toward these inhibitors as other known LMW PTPS (Soulat et al., 2002; Koul et al., 2000; Grangeasse et al., 1998; Li and Strohl, 1996; Tonks et al., 1988). Ammonium molybdate, sodium orthovanadate, and \(\text{Zn}^{2+}\) are known to be potent inhibitors of PTPs (Tonks et al., 1988; Grangeasse et al., 1998; Soulat et al., 2002), and were found to strongly inhibit the activity of SynPTP. Sodium fluoride, a non-specific inhibitor (Tonks et al., 1988; Koul et al., 2000) for phosphatases, was found to be without inhibitory effect on SynPTP. Okadaic acid, a potent inhibitor (Shi et al., 1999) of many protein serine/threonine phosphatases (PP2A), failed to inhibit the catalytic activity of SynPTP. Results are summarized on Table 3-7.
Table 3-7. Influence of various phosphatase inhibitors on the catalytic activity of SynPTP.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Concentration (mM)</th>
<th>specific activity (pmol/min/mg)</th>
<th>relative activity (% RCML-$^{32}$P-Tyr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>560 ± 20</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium Molybdate</td>
<td>5</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td>Sodium Orthovanadate</td>
<td>5</td>
<td>44 ± 2.5</td>
<td>8</td>
</tr>
<tr>
<td>Zinc Sulphate</td>
<td>5</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td>Sodium Sulfate</td>
<td>5</td>
<td>640 ± 35</td>
<td>114</td>
</tr>
<tr>
<td>Sodium Fluoride</td>
<td>5</td>
<td>680 ± 14</td>
<td>120</td>
</tr>
<tr>
<td>Okadaic acid</td>
<td>0.005</td>
<td>720 ± 22</td>
<td>128</td>
</tr>
</tbody>
</table>

All assays were carried out as described in Materials and Methods. RCML($^{32}$P-Tyr) was used as substrate at a final concentration of 2µM of protein bound phosphate. Potential inhibitors were present at the indicated final concentration. The enzyme activity is reported relative to that observed with RCML($^{32}$P-Tyr) in the absence of any inhibitor, which was 560 pmol/min/mg, and set equal to 100%. ND* represents not detectable, less than 2%, or 12 pmol/min/mg.
Summary of Specific Aim-2:

It was observed that SynPTP displays phosphohydrolase activity toward $\beta$-napthyl phosphate or free phosphotyrosine, but not toward $\alpha$-napthyl phosphate or phosphoserine/phosphothreonine. Protein phosphatase activity of SynPTP was detected toward tyrosine phosphorylated protein substrates. However, it was observed that SynPTP was unable to dephosphorylate serine phosphorylated protein substrates. Further it was observed that the catalytic activity of SynPTP was inhibited by many potent protein tyrosine phosphatase inhibitors (sodium orthovanadate, ammonium molybdate, Zn$^{2+}$), but not by okadaic acid or sodium fluoride, which are inhibitors of many serine/threonine phosphatases.
Specific Aim-3: To identify physiologically relevant enzyme-substrate relationships between SynPTP and phosphoprotein substrate(s) from *Synechocystis* sp. PCC 6803.

Goals:

i) Isolate potential substrates for SynPTP from *Synechocystis* sp. PCC 6803 by substrate trapping.

ii) Determine whether proteins isolated by substrate trapping are phosphorylated on tyrosine.

Members of the protein tyrosine phosphatase (PTPase) family share a common mechanism for phosphomonoester hydrolysis (Zhang, 1998; Wu and Zhang, 1996; Denu et al., 1996) (Figure 1-5). Detailed studies have shown that mutagenic alteration of either the catalytic cysteine, conserved aspartate, or both renders a PTPase incapable of dephosphorylating substrates. However, these mutagenically altered PTPases still retain the ability for binding substrates (Agazie and Hayman, 2003; Jeon et al., 2002; Zhang, et al., 2003, 2000; Xie et al., 2002). Consequently, these mutationally altered forms of PTPases have been named “Substrate Trapping Mutant(s)” (Garton et al., 1996) and are commonly used as affinity reagents to isolate and identify physiological substrates for various PTPases.

Mutagenically altered Cys7Ser and Asp125Ala SynPTP (alteration of both residues) was used as a “Substrate Trapping Mutant” to isolate potential substrates for SynPTP from cell extracts of *Synechocystis* sp. PCC 6803. Active
SynPTP was used as a negative control. *Synechocystis* sp. PCC 6803 cell extracts, either membrane fractions or soluble fractions, were applied to columns (Amersham Biosciences, Sweden), on which either active SynPTP or mutagenically altered inactive SynPTP had been immobilized as described in Materials and Methods. Bound proteins were eluted from these columns with a step gradient of 50 mM to 1 M NaCl. Portions of the eluted material from both columns (active SynPTP column and mutagenically altered inactive SynPTP column) were analyzed by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody. The results of the immunodetections were compared between the fractions eluted from active SynPTP column and inactive mutagenically altered SynPTP column. Potential phosphotyrosine proteins that were present in the immunoblot from the eluted fractions of inactive SynPTP column but not the active SynPTP column were considered potential substrates.

**Analysis of Soluble Fractions:**

Soluble fraction containing 15 mg protein in 10 ml of supernatant was applied to phosphatase bound Sepharose column (either active SynPTP or mutagenically altered SynPTP) and bound proteins were eluted with a step gradient of 50 mM to 1 M NaCl (50 mM, 100 mM, 150 mM, 200 mM, 250 mM, 250 mM with 50 mM free phosphotyrosine, and 1 M with 50 mM free phosphotyrosine) as described in Materials and Methods. Eluted fractions for each individual step, 2 ml, were collected and stored at 4°C.
A portion from each step was analyzed by SDS-PAGE and immunodetection was performed using an anti-phosphotyrosine antibody. No immunoreaction was observed in 50 mM or 150 mM eluates (data not shown). However, immunodetection for phosphotyrosine-containing proteins was observed in 200 mM NaCl, 250 mM NaCl, and 250 mM NaCl plus 50 mM free phosphotyrosine eluates (Figure 3-10).

The presence of two putative phosphotyrosine containing proteins in the molecular mass range of 15 kDa to 20 kDa was observed in the eluates of 200 mM NaCl and 250 mM NaCl from mutagenically altered SynPTP column (Figure 3-10D, Lane: 2,4). These two proteins were not observed in the eluates of either 200 mM NaCl or 250 mM NaCl (Figure 3-10D, Lane:1,3) from active SynPTP column. Therefore, These two proteins were considered potential substrates for SynPTP.
Figure 3-10. Identification of potential protein substrates for SynPTP from soluble fractions of *Synechocystis* sp. PCC 6803. Soluble fractions of *Synechocystis* cell extracts were applied to active SynPTP bound Sepharose or mutagenically altered inactive SynPTP bound Sepharose. Bound proteins were eluted with 200 mM NaCl, 250 mM NaCl, and 250 mM NaCl containing 50 mM phosphotyrosine. Eluted proteins were subjected to SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody. (A) Shown is the analysis of SDS-PAGE for soluble fractions of *Synechocystis* cell extracts. Gel was stained with Coomassie blue. Lane 1: Protein Marker, Lane 2: 15 µg of total protein from the soluble fractions of *Synechocystis* cell extracts. (B) Indicates the corresponding result of Western blot analysis of a duplicate gel. Immunoblot was probed with anti-phosphotyrosine antibody. Arrowheads indicate the positions of prominently immunoreactive proteins. (C) Indicates the results of substrate trapping experiment, analyzed by SDS-PAGE. Gel was stained with Coomassie blue. Lane 1, 3, 5 contained proteins eluted from active SynPTP column with 200 mM NaCl, 250 mM NaCl, and 250 mM NaCl with 50 mM phosphotyrosine respectively. Lane 2, 4 6 contained proteins eluted from mutagenically altered inactive SynPTP column with 200 mM NaCl, 250 mM NaCl, and 250 mM NaCl with 50 mM phosphotyrosine respectively. (D) Represents the corresponding result of Western blot analysis of a duplicate gel from SDS-PAGE. A monoclonal anti-phosphotyrosine antibody conjugated with horseradish peroxidase was used to detect phosphotyrosine containing proteins eluted from the active SynPTP column or mutagenically altered inactive SynPTP column. Arrowheads indicate the positions of potential substrates for SynPTP.
The portions of the Coomassie blue stained gel containing each protein (indicated by arrowheads in Figure 3-10C) were excised, treated with trypsin as described in Materials and Methods, and peptides were extracted. Extracted peptides were analyzed by mass spectrometry. The fractions, eluted from the active SynPTP or mutagenically altered inactive SynPTP bound Sepharose with 250 mM NaCl, were also treated with trypsin for mass spectral analysis. A nano-electrospray quadrupole ion trap mass spectrometer was used for these analyses. Mass peptide profiles were matched to the potential proteins sources using NCBI nr (http://www.ncbi.nlm.nih.gov/BLAST) database through Mascot search developed by www.matrixscience.com. The results for both analyses best matched the following proteins from *Synechocystis* sp. PCC 6803, which are: i) Sll1577 (CpcB), β subunit of phycocyanin, molecular weight 18.8 kDa, accession no. 1652309 ii) Sll1578 (CpcA), α subunit of phycocyanin, molecular weight 17.8 kDa, accession no. 1652308. The results of mass spectral analysis are summarized in Table 3-8, 3.9 and 3-10. Mowse score denotes probability based Molecular weight search of peptide masses. A Mowse search is a comparison of the calculated peptide masses for each entry in the sequence database with the set of experimental data. Each calculated value, which falls within a given mass tolerance of an experimental value, counts as a match. Mowse score is an addition of individual ions score. Ions score is $-10\times \log(P)$, where $P$ is the probability that the observed match is a random event (www.matrixscience.com).
Table 3-8. A mass peptide profile of potential substrates for SynPTP, analyzed from substrate trapping experiment by a nano-electrospray quadrupole ion trap mass spectrometer.

<table>
<thead>
<tr>
<th>No of peptide</th>
<th>Peptide mass observed (Da)</th>
<th>Peptide mass calculated (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>914.59</td>
<td>914.43</td>
</tr>
<tr>
<td>2</td>
<td>1691.98</td>
<td>1693.82</td>
</tr>
<tr>
<td>3</td>
<td>2099.87</td>
<td>2099.04</td>
</tr>
<tr>
<td>4</td>
<td>1464.04</td>
<td>1465.72</td>
</tr>
<tr>
<td>5</td>
<td>1467.25</td>
<td>1467.77</td>
</tr>
<tr>
<td>6</td>
<td>1532.37</td>
<td>1531.75</td>
</tr>
<tr>
<td>7</td>
<td>1598.55</td>
<td>1596.81</td>
</tr>
<tr>
<td>8</td>
<td>1654.77</td>
<td>1654.82</td>
</tr>
<tr>
<td>9</td>
<td>1816.09</td>
<td>1815.97</td>
</tr>
<tr>
<td>10</td>
<td>2728.02</td>
<td>2729.39</td>
</tr>
<tr>
<td>11</td>
<td>1906.90</td>
<td>1906.89</td>
</tr>
<tr>
<td>12</td>
<td>2897.10</td>
<td>2895.36</td>
</tr>
<tr>
<td>13</td>
<td>2109.17</td>
<td>2109.02</td>
</tr>
</tbody>
</table>

Mass spectral analysis was performed using the fractions, eluted from the mutagenically altered inactive SynPTP bound Sepharose with 250 mM NaCl and treated with trypsin as described in Materials and Methods.

Table 3-9. A proteomic analysis of potential substrates for SynPTP, observed from substrate trapping experiment, in soluble fractions of *Synechocystis* sp. PCC 6803 cell extracts.

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>No. of peptides matched/total</th>
<th>Mowse Scorea</th>
<th>Predicted Mr</th>
<th>Observed Mr</th>
<th>% Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phycocyanin β</td>
<td>5/13</td>
<td>250</td>
<td>18.8</td>
<td>19</td>
<td>45</td>
</tr>
<tr>
<td>Phycocyanin α</td>
<td>3/13</td>
<td>180</td>
<td>17.8</td>
<td>17</td>
<td>25</td>
</tr>
</tbody>
</table>

a Mowse score is described in text. Predicted molecular mass was deduced from amino acid sequence, calculated by using the PROTEIN CALCULATOR v3.2 program (http://www.scripps.edu/~cdputnam/protcalc.html). Observed molecular mass was determined from Coomassie blue stained SDS-gel.
Table 3-10. Partial sequence analysis of ~19 kDa and 17 kDa proteins, potential substrates for SynPTP, by mass spectral analysis.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptide no.</th>
<th>Observed mass (Da)</th>
<th>Calculated mass (Da)</th>
<th>Sequence</th>
<th>Individual ions scorea</th>
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<tr>
<td>Phycocyanin β</td>
<td>1</td>
<td>914.59</td>
<td>914.53</td>
<td>MFDVFTR.V</td>
<td>34</td>
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<tr>
<td></td>
<td>13</td>
<td>2109.17</td>
<td>2109.02</td>
<td>R.GEYLQALDSLATSVAEGNK.R</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>1906.90</td>
<td>1906.89</td>
<td>R.YVTYATFTGADASVLED.R.C</td>
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<tr>
<td></td>
<td>9</td>
<td>1816.09</td>
<td>1815.97</td>
<td>R.ETYVAGVGASVAAGVQK.R</td>
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</tr>
<tr>
<td></td>
<td>7</td>
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<td>1596.81</td>
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</tr>
<tr>
<td>Phycocyanin α</td>
<td>5</td>
<td>1467.25</td>
<td>1467.77</td>
<td>R. FLSSTELQIAFGR.L</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>6</td>
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<td>1531.75</td>
<td>K. TPLTEAVSTADSQGR.F</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1654.77</td>
<td>1654.82</td>
<td>R.TFDLSPSYVEALK.Y</td>
<td>54</td>
</tr>
</tbody>
</table>

a Individual ions score is described in text.

Shown are the partial sequences of amino acids deduced from peptides mass fingerprint obtained after fragmentations of tryptic peptides by MS/MS analysis. Observed mass and calculated mass of each peptide was determined from Mascot search results of www.matrixscience.com. Individual ions score > 30 indicated homology. Individual ions score > 52 indicated identity or extensive homology.

Phycocyanin β:

1 MFDVFTRVVS QADARGEYLS GSQLDALSAT VAEGNK RIDS VNRITGNASA
51 IVSNAARALF AEQPQLIQPG GNAYTSKRMA ACLRDMEIL RYVTYATFTG
101 DASVLEDRC RQANAGLQA AKALTDNAQN
151 GDCSAIVAEI AGYFDRAAAA VA

Phycocyanin α:

1 MKTPLTEAVS TADSQGFLS STELQIAFGR LRQANAGLQA AKALTDNAQN
51 LVNGAQQAVY NKPPYTTTQ GNNFAADQRG KDKCARDIGY YLRIVTYCLV
101 AGGTGPLDEY LIAGIDEINR TFDLSPSWV EALKYIKANH GLSGDARDEA
151 NSYLDYAINA LS

Figure 3-11. Matched peptides for β and α subunits of phycocyanin are shown in bold red.
However, no tyrosine phosphorylated peptide was detected by mass spectral analysis. Therefore, we performed additional experiments to determine whether phycocyanin contains phosphotyrosine. Phycocyanin was isolated from soluble fractions of *Synechocystis* sp. PCC 6803 cell extracts by anion exchange column chromatography or size exclusion column chromatography. 2D gel electrophoresis was performed to separate phycocyanin from other proteins present in soluble fractions of cell extracts. In addition, purified fraction of phycocyanin was incubated with active SynPTP and probed with anti-phosphotyrosine antibody to determine whether the source of immunoreactivity could be eliminated through the interaction of a protein tyrosine phosphatase.

**Purification of Phycocyanin from Soluble Fractions of *Synechocystis* sp. PCC 6803 Cell Extracts by Anion Exchange Chromatography:**

Phycocyanin was isolated from soluble fractions of *Synechocystis* sp. PCC 6803 by anion exchange chromatography as described in Materials and Methods. Fractions eluted from the column were analyzed by SDS-PAGE and immunodetection was performed using anti-phosphotyrosine antibody. No phosphotyrosine containing proteins were observed in the range of 15 kDa to 20 kDa (Molecular mass of α and β subunits of phycocyanin are in the range of 15 kDa to 20 kDa) from the fractions that eluted from the column with 75 mM or 100 mM NaCl. However, immunoreactivity was detected from the fractions eluted with 125 mM to 175 mM NaCl. Moreover, the sizes of these proteins matched that
expected for phycocyanin. Arrowheads in Figure 3-12 indicate the probable positions of phycocyanin β and α subunits.

Portions of the Coomassie stained gel containing these proteins were excised, treated with trypsin, peptides were extracted, and extracted peptides were subjected to mass spectral analysis. The results matched phycocyanin β for the top band with a Mowse score 231 and phycocyanin α for the bottom band with a Mowse score 177. The definition of a Mowse score was described in the previous section.
Figure 3-12. Immunodetection of phosphorylated phycocyanin from the soluble fraction of *Synechocystis* sp. PCC 6803 purified by anion exchange column chromatography. (A) Indicates SDS-PAGE analysis for purified fractions of phycocyanin eluted from DE-52 anion exchange column. Lane 1: protein molecular weight markers; Lane 2,3: Fractions were eluted by 75 mM [NaCl], Lane 4,5: Fractions were eluted by 100 mM [NaCl]. Total amount of protein loaded to lane 2 to lane 5 was 6.5µg. (B) Indicates the result of western blot analysis of corresponding gel. Anti-phosphotyrosine antibody was used to detect the presence of tyrosine phosphorylated phycocyanin. (C) Shows the result of SDS-PAGE analysis of the purified fractions of phycocyanin eluted by 125 mM to 175 mM [NaCl]. Lane 1: protein molecular weight markers; Lane 2: 15 µg of total protein in soluble fractions of Synechocystis cell extracts; Lane 3,4: fractions, eluted by 125 mM [NaCl]; Lane 5,6: Proteins were eluted by 150 mM of [NaCl]; Lane 7,8: Fractions containing the elutions with 175 mM [NaCl]. The amount of protein, loaded to lanes 3 to 8 was 6.5µg. Arrowheads indicate the position of phycocyanin β subunit (top band, 18.9 kDa) and phycocyanin α subunit (lower band, 17.8 kDa). (D) Indicates the corresponding result of Western blot analysis of the duplicate gel.
2D Gel Electrophoresis of Phycocyanin:

Next, the pI of those phosphotyrosine proteins (Figure 3-12) was determined and compared with the pI of phycocyanin \( \beta \) and \( \alpha \) subunits. Since, tyrosine phosphorylated phycocyanin \( \alpha \) and \( \beta \) were detected from those fractions that eluted from anion exchange chromatography with 125 mM NaCl to 175 NaCl, these fractions were mixed together and analyzed by 2D gel electrophoresis.

The procedure was followed as described in Materials and Methods. A 7 cm long IPG (Immobilin pH gradient) strip, pH 4-7 linear, was used. The total amount of protein loaded to each strip was 125 \( \mu \)g. After the second dimension, proteins were transferred to a PVDF membrane and immunodetection was performed using the anti-phosphotyrosine antibody, RC20H (BD Science). The predicted pI of phycocyanin \( \beta \) subunit is 5.1 and phycocyanin \( \alpha \) subunit is 5.5. After immunodetection the pI of phycocyanin \( \beta \) and \( \alpha \) was observed to range from 4.5 to 5.5. The arrowhead in Figure 3-13 indicates the predicted position of phycocyanin. The observed pI values for phycocyanin \( \beta \) and \( \alpha \) were compared with those values previously determined by Wang and Chitnis, 2000 (Table 3-11).
Table 3-11. Comparison of deduced pl and observed pl of phycocyanin

<table>
<thead>
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<th>Protein ID</th>
<th>Deduced pl</th>
<th>Observed pl</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Phycocyanin β</td>
<td>5.1</td>
<td>4.5 to 5.5</td>
<td>This report</td>
</tr>
<tr>
<td>Phycocyanin β</td>
<td>5.0</td>
<td>4.6</td>
<td>Wang and Chitnis, 2000</td>
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<tr>
<td>Phycocyanin α</td>
<td>5.5</td>
<td>4.5 to 5.5</td>
<td>This report</td>
</tr>
<tr>
<td>Phycocyanin α</td>
<td>5.4</td>
<td>4.9</td>
<td>Wang and Chitnis, 2000</td>
</tr>
</tbody>
</table>

Deduced pl was determined from amino acid sequence, calculated by using the PROTEIN CALCULATOR v3.2 program (http://www.scripps.edu/~cdputnam/protcalc.html). Observed pl was determined from Coomassie blue stained acrylamide gel of 2D gel electrophoresis.

Figure 3-13. 2D gel electrophoresis of phycocyanin. Fractions, that eluted from anion exchange resin with 125 mM NaCl to 175 mM NaCl and were immunoreactive with anti-phosphotyrosine antibody, were mixed together. Total amount of these phosphotyrosine containing proteins applied to each IPG strip was 125 µg. (A) Indicates the Coomassie blue stained gel. (B) Represents the result of Western blot analysis of a duplicate gel. Immunodetection was performed as described in Materials and Methods using a monoclonal antibody conjugated with horseradish peroxidase. Arrowheads indicate the position of phycocyanin.
Purification of Phycocyanin by Size Exclusion Chromatography:

Besides phycocyanin, many other putative phosphotyrosine proteins were observed in the immunoblot with apparent molecular weights greater than 20 kDa. We focused on two phosphotyrosine proteins in the molecular range of 15~20 kDa for our study. Consequently the higher molecular weight phosphotyrosine proteins were not identified. Further purification of phycocyanin by size exclusion chromatography was performed using the fractions obtained from anion exchange chromatography in an effort to eliminate other proteins of higher molecular weight (above 20 kDa) from the mixture. If a pure fraction of phycocyanin can be obtained from size exclusion chromatography in the molecular size range of 15~20 kDa range and if this fraction does immunoreact with anti-phosphotyrosine antibody, it will implicate phycocyanin as a tyrosine phosphorylated protein.

Purification was performed as described in Materials and Methods. The blue colored fractions, whose ratio of $A_{620}/A_{280}$ was in the range of 3.8 to 4.2 (phycocyanin-rich fractions), were analyzed on SDS-polyacrylamide gel. Western blot analysis was performed using anti-phosphotyrosine antibody (Figure 3-14). For each fraction, two protein bands were observed on the SDS-gel in the molecular size range of 15~20 kDa (looks like one band on the scanned picture), the expected molecular size of phycocyanin $\beta$ and $\alpha$ subunits. It was also observed that these fractions were immunoreactive with the anti-phosphotyrosine antibody. Results indicated that these two proteins are tyrosine phosphorylated.
Figure 3-14. Purification of phycocyanin by size exclusion column chromatography. Each lane contains approximately 6 µg of protein. (A) Shown is the analysis of those fractions, which contain a ratio of A_{620}/A_{280} in the range 3.8 to 4.2, on 12% (w/v) SDS-gel stained with Coomassie blue. (B) Corresponding results of immunodetection of the duplicate gel. Immunoblots were probed with anti-phosphotyrosine antibody. Arrowheads indicate the expected position of phycocyanin.

The fractions which reacted with anti-phosphotyrosine antibody were mixed together. A portion of the mixture containing 25 µg of protein, was applied for 2D gel electrophoresis (Figure 3-15). It was observed that the position of phycocyanin matched that previously observed (Figure 3-13). Immunodetection of the duplicate gel of 2D gel electrophoresis was not performed, since it already had been observed (Figure 3-14) that these mixed fractions (fractions eluted from size exclusion chromatography) were reactive toward anti-phosphotyrosine antibody.
Figure 3-15. 2D gel electrophoresis of purified phycocyanin eluted from size exclusion chromatography. The fractions, which has $A_{620}/A_{280}$ ratio in the range of 3.8 to 4.2 and reacted with anti-phosphotyrosine antibody, were mixed together. A 7 cm long IPG strip, pH 4-7 linear was used. The total amount of protein loaded to the IPG strip was 25 µg. After the second dimension, the gel was stained with silver stain. Arrowhead indicates the position of phycocyanin.

It was reported that phycocyanin autofluoresces due to the covalent attachment of bilin groups, which are open-chain tetrapyrroles (Grossman et. al., 1993; MacColl and Robert 1998; Piven et al., 2005). Therefore, we asked whether the signal obtained during immunodetection of phycocyanin with anti-phosphotyrosine antibody might have arisen due to the fluorescence of phycocyanin rather than chemiluminescence from the reporter enzyme of antibody.
The purified fraction of phycocyanin eluted from anion-exchange resin were resolved on a 12% (w/v) SDS-gel and the proteins were transferred to a PVDF membrane. Following that, an X-ray film was exposed to the membrane under different conditions and the film was developed. First, film was developed after exposure to a dry membrane. Second, film was exposed to the wet membrane in TBS buffer (Tris-base saline, pH 7.5) and the corresponding film was developed. Third, a duplicate wet membrane from second condition was incubated with ECL reagent (Pierce, Rockford III) for 5 minutes then exposed to the film. Fourth, the membrane was incubated with an anti-phosphotyrosine antibody conjugated with horseradish peroxidase and immunodetection was performed as described in Materials and Methods. Fifth, the anti-phosphotyrosine antibody was pre-incubated with 5 mM free phosphotyrosine for 1 hour at room temperature, then immunodetection was carried out as described in Materials and Methods. No luminescent band for phycocyanin was visualized after exposing the film under the first three conditions (Figure 3-16 A, B, C). Protein band for phycocyanin was only visualized when the membrane was incubated with anti-phosphotyrosine antibody, which indicated that only the chemiluminescence of immunoreactive species was detected (Figure 3-16D). The immunoreactivity of the antibody was blocked when it was pre-incubated with 5 mM free phosphotyrosine.
Figure 3-16. Comparison of the intensity of the intrinsic autofluorescence of phycocyanin with Chemiluminescence produced by Western blot analysis. Purified phycocyanin eluted from anion exchange column was analyzed on SDS-gel and proteins were transferred to a PVDF membrane. (A) Shows the picture of a X-ray film exposed on the dry membrane. (B) shown is the picture of the wet membrane in TBS buffer, which was exposed to the x-ray film. (C) shows a duplicate of the wet membrane that was incubated with ECL reagent and then exposed to the X-ray film. (D) Shows the immunoblot probed with anti-phosphotyrosine antibody. (E) Shows the Immunoblot that was prepared with pre-incubated anti-phosphotyrosine antibody in presence of 5mM free phosphotyrosine.
Determination of the Specificity of Anti-Phosphotyrosine Antibody toward Phosphotyrosine Epitope:

Next we examined the specificity of the anti-phosphotyrosine antibody, RC20, used for all immunodetection experiments to detect phosphotyrosine proteins. We questioned whether this antibody was specifically binding to a phosphotyrosine epitope during immunoreactions. We asked whether immunoreactivity could be competed with free phosphotyrosine, free tyrosine, or free phosphoserine.

Fractions containing purified phycocyanin from size exclusion column chromatography were resolved on a 12% (w/v) SDS-gel and transferred to a PVDF membrane. The membrane was probed with anti-phosphotyrosine antibody or antibody that had been pre-incubated with either phospho-serine, phospho-tyrosine, or tyrosine (1 mM each). Protein bands were visualized using enhanced Chemiluminescence reagents (Pierce, Rockford III). It was observed that the intensity of signal for immunodetection of phycocyanin was abolished only when membrane was probed with antibody that had been pre-incubated with 1 mM free phosphotyrosine (Figure 3-17D). Immunoreactivity of the antibody was not blocked when it was pre-incubated with free tyrosine or phosphoserine (Figure 3-17B, C), and appeared to be similar in intensity to the immunoblot that had been (Figure 3-17A) incubated with antibody alone.
Figure 3-17. Western blot analysis of phycocyanin for competition of antibody with free phosphoamino acids. Purified phycocyanin, obtained from size exclusion column chromatography, 6 µg, was resolved by SDS-PAGE and transferred to a PVDF membrane. Immunodetection with anti-phosphotyrosine antibody was performed as described in Materials and Methods. Antibody was pre-incubated at room temperature for 1 hour with 1 mM specified compound as mentioned below. (A) no added phosphoamino acids. (B) 1 mM tyrosine. (C) 1 mM phospho-serine. (D) 1 mM phospho-tyrosine.
Detection of Phosphorylated/Dephosphorylated Phycocyanin by Immunodetection following Incubation without or with Active SynPTP:

Next we addressed the question of whether recombinant SynPTP is able to dephosphorylate phycocyanin as would be expected if it contained phosphotyrosine. If so, the anti-phosphotyrosine antibody will be able to recognize tyrosine phosphorylated phycocyanin but not dephosphorylated phycocyanin.

Purified phycocyanin, 125 µg, obtained either from anion exchange column or size exclusion column chromatography, was incubated with or without 1 µg active recombinant SynPTP or without active SynPTP (as a positive control) at 30°C for 1 hour. The reaction mixture consisted of 100 mM MES, pH 6.5, 1 mM EDTA, and 2 mM DTT in a 200 µL volume. After incubation, the reaction mixture was passed through a 0.5 ml bed volume of Ni⁺² column. Since, recombinant SynPTP contains a histidine tag, it was expected that SynPTP would bind to the Ni⁺² column and phycocyanin would flow through. Bound SynPTP was eluted from the column using 250 mM imidazole in 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 5% (v/v) glycerol. A portion of the flow through and imidazole eluant from the column was analyzed on 12% (w/v) SDS-polyacrylamide gel, transferred to the PVDF membrane and subjected to immunodetection. It was noticed that immunoreactivity of antibody was abolished when phycocyanin was incubated with SynPTP as compared to phycocyanin alone (Figure 3-18).
Figure 3-18. Immunodetection of phosphorylated/dephosphorylated phycocyanin after incubation without/with SynpTP. The procedure for immunodetection was followed as described in Materials and Methods. Approximately 3.5 to 5 µg of total protein was loaded into each lane. (A) Analysis of SDS-PAGE stained with Coomassie blue. Lane 1: Molecular weight markers; Lane 2: Approximately 3.5 µg of SynpTP after elution from Ni\(^{+2}\) column, as described in text; Lane 3,4: Approximately 4.5 µg of phycocyanin, obtained from anion exchange column, collected from the flow through of the Ni\(^{+2}\) columns as described in text. Lane 5: Approximately 5 µg of phycocyanin (obtained from anion exchange column) incubated alone without SynpTP as a positive control and passed through Ni\(^{+2}\) column. Lane 6,7: approximately 4 µg of phycocyanin, obtained from gel filtration column, collected from the flow through of the Ni\(^{+2}\) column, as described in text. Lane 8: Approximately 5 µg of phycocyanin, obtained from gel filtration column, incubated alone without SynpTP as a positive control and passed through Ni\(^{+2}\) column. (B) Represents the corresponding result of Western blot analysis from a duplicate gel, probed with an anti-phosphotyrosine antibody.
Comparison of Intensity of Immunoreactivity for Phycocyanin in Cell extracts of Wild type and a Phycocyanin-less Mutant Strain of *Synechocystis* sp. PCC 6803:

Next, we compared the intensity of the immunoreactivity of antibody for phycocyanin between wild type strain and a phycocyanin less mutant strain (CK mutant) of *Synechocystis* sp. PCC 6803. If phycocyanin is not present in the mutant strain, then signal for immunoreaction of antibody with phycocyanin will be weak or less than that of wild type strain.

A mutant strain of *Synechocystis* sp. PCC 6803, which lacks the genes encoding phycocyanin α subunit (CpcA, Sll1577), phycocyanin β subunit (CpcB, Sll1578), 33 kDa rod linker protein (Cpc1, Sll1580), and 30 kDa rod linker protein (Cpc2, Sll1579) was obtained from Cyanobase. This mutant strain is called the CK mutant (Ughy and Ajlani, unpublished work). The wild type strain was grown in BG-11 media under normal growth conditions as described in Materials and Methods. The CK mutant strain was grown under similar conditions to wild type strain with 10µg/ml kanamycin added as a selection marker. Both wild type and mutant strains were cultured starting at an OD$_{730\text{nm}}$ = 0.03 and cultivated for 20 days. At that point the OD$_{730\text{nm}}$ for wild type strain reached 1.0. However, the OD$_{730\text{nm}}$ of CK mutant cells was not measured. It was observed that the growth of CK mutant strain was poor in comparison to wild type strain (Figure 3-19). The cells were harvested by centrifugation at 2700xg for 20 min. Whole cell extracts for both wild type and CK mutant strains were prepared as described in Materials and Methods with the exception that cell lysis buffer included 0.1mM sodium orthovanadate, 0.1% Tween-20 (v/v), and 0.1% Triton-X-100 (v/v). A portion of
whole cell extracts from both wild type and CK mutant, 30 µg protein each, were resolved on 12% (w/v) SDS-PAGE and immublotted with anti-phosphotyrosine antibody (Figure 3-20).

Figure 3-19. Culture of wild type and CK mutant strain of *Synechocystis* sp. PCC 6803. Cells were grown under white light intensity at 10 µmol photons m\(^{-2}\)s\(^{-1}\) for 20 days at continuous shaking with 100 rpm as described in text.

It was noticed that the intensity of immunoreactivity of antibody for phycocyanin (position of phycocyanin was indicated by arrowhead) was much weaker in mutant strain than that in the wild type strain (Figure 3-20).
Figure 3-20. Comparison of intensity for immunoreactivity of anti-phosphotyrosine antibody toward phycocyanin in wild type and CK mutant strain of *Synechocystis* sp. PCC 6803. Each lane contains 30 µg of total proteins. (A) shown is the analysis of SDS-PAGE, stained with Coomassie blue. Lane 1: molecular weight protein markers; Lane 2: empty; Lane 3: whole cell extracts of wild type *Synechocystis* sp. PCC 6803; Lane 4: empty; Lane 5: whole cell extracts of CK mutant strain of *Synechocystis* sp. PCC 6803. (B) shows the corresponding Western blot analysis using anti-phosphotyrosine antibody. Arrowheads indicate the estimated position of phycocyanin.
Analysis and Comparison of Immunoreactivity of Anti-Phosphotyrosine Antibody with Phycocyanin among the Cells of Wild Type, PAL Mutant and Cells Grown under Nutrient-deprivation:

It was reported that, in *Synechocystis* sp. PCC 6803, phycobilisome proteins are degraded under a variety of nutrient-deficient conditions. The degradation of phycobiliproteins causes the color of the culture to change from blue green to yellow. This process is called chlorosis or bleaching (Li and Sherman, 2002; Tandeau de Marsac and Houmard, 1993; Dolganov and Grossman, 1999; Schwarz and Grossman, 1998; Colier and Grossman, 1994). To further examine whether phycocyanin is tyrosine-phosphorylated, *Synechocystis* sp. PCC 6803 cells were cultured in BG-11 media under nitrogen or phosphate deprived condition. The immunoreactivity of the anti-phosphotyrosine antibody with phycocyanin was compared among wild type cells and the cells cultured under nutrient deprived conditions. If phycocyanin is the source of immunoreactivity then the intensity of immunoreactivity of the antibody should be diminished, when phycocyanin is degraded under nutrient deprived condition, in compare to wild type strain.

In addition, another mutant strain of *Synechocystis* sp. PCC 6803, which lacks phycocyanin, allophycocyanin, and the core membrane linker protein (Ajlani and Vernotte, 1998), was obtained from Cyanobase. This mutant strain is called PAL mutant. We also analyzed the intensity of immunoreaction of antibody with phycocyanin in this mutant strain and compared that with wild type strain. If
phycocyanin is not present in the mutant strain, then there should be no immunoreactivity of antibody for phycocyanin in comparison to wild type strain.

Wild type *Synechocystis* sp. PCC 6803 was cultured in BG-11 media under normal growth conditions as described in Materials and Methods. The PAL mutant strain was cultured in BG-11 media under similar conditions to wild type strain in presence of 10 µg/ml chloramphenicol as a selection marker. Wild type cells were also cultivated in BG-11 media lacking sodium nitrate as a nitrogen source. For phosphate depletion, cells were cultured in BG-11 media in the absence of di-potassium hydrogen phosphate. Wild type cells, PAL mutant cells and wild type cells under nutrient deprived conditions were initially cultured to an initial OD$_{730}$nm = 0.025 and cultivated for 7 days. Cells were cultured until the OD$_{730}$nm for wild type strain reached to 0.8. However, the OD$_{730}$nm of the PAL mutant cells or cells cultured under nutrient deprived conditions were not measured. It was noticed that cells for PAL mutant or under nutrient deprived conditions grew very poorly as compared to wild type cells (Figure 3-21).

Whole cell extracts from wild type, PAL mutant strain, and wild type cells grown under nitrogen and phosphate starvation, were prepared as described in Materials and Methods with the exception that cell lysis buffer included 0.1mM sodium orthovanadate 0.1% Tween-20 (v/v), and 0.1% Triton-X-100 (v/v). Whole cell extracts, containing ~9 µg each, were analyzed on 15% (w/v) SDS-PAGE and immublotted with anti-phosphotyrosine antibody (Figure 3-21). It was observed that there was almost no immunoreactivity of antibody toward phycocyanin in the cells grown under nitrogen depletion (Figure 3-22, Lane 2), or
cells from PAL mutant strain (Figure 3-22, Lane 5). Very little immunoreactivity was detected from the cells grown under phosphate deprivation (Figure 3-22, Lane 3).

Figure 3-21. Culture of WT *Synechocystis* sp. PCC 6803 under different growth conditions and PAL mutant strain of *Synechocystis* sp. PCC 6803. Cells were grown under white light of intensity 10 µmol photons m⁻²s⁻¹ for 7 days at continuous shaking with 100 rpm, until OD₇₃₀nm for wild type reached 0.8 (log phase) as described in text. (A) PAL mutant strain of *Synechocystis* sp. PCC 6803. (B) Flask 1: cells were grown under nitrogen starvation; Flask 2: cells were grown under normal growth condition; Flask 3: cells were grown under phosphate starvation.
Figure 3-22. Comparison of immunoreactivity with anti-phosphotyrosine antibody toward phycocyanin among the cell extracts from WT, PAL mutant, nitrogen deprived, and phosphate deprived cells of *Synechocystis* sp. PCC 6803. Whole cell extracts of *Synechocystis* sp. PCC 6803, grown as described in the text, were analyzed by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody. (A) Shown is the result of SDS-PAGE on a 15% (w/v) polyacrylamide gel, stained with Coomassie blue. Each lane contains approximately 9 µg of total protein. Lane 1: Molecular weight markers; Lane 2: Cells were grown under nitrogen depletion in media; Lane 3: cells were grown under phosphate starvation; Lane 4: empty, Lane 5: Pal mutant strain; Lane 6: empty; Lane 7: WT strain. (B) Represents the corresponding result of Western blot analysis from the duplicate gel of SDS-PAGE. Immunoblot was probed with anti-phosphotyrosine antibody as described in Materials and Methods. Arrowheads indicate the predicted position of phycocyanin.
Detection of Potential Substrates for SynPTP \textit{in vitro} from Membrane Fractions of \textit{Synechocystis} sp. PCC 6803 Cell Extracts by Substrate Trapping Experiments:

Preparation of membrane fractions was performed as described in Materials and Methods. Membrane fractions were solubilized with Triton-X-100 in buffer containing 50 mM MES (pH 6.5), 10 mM NaCl, 1 mM DTT, 1 mM EDTA, and protease inhibitor cocktail. Substrate trapping experiment was performed with membrane fractions as described for the analysis of soluble fractions. A portion of the material eluted from the columns was analyzed by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody (Figure 3-23). Several putative phosphotyrosine containing proteins were detected from the eluates obtained using 200 mM NaCl containing 50 mM phosphotyrosine, collected from mutagenically altered SynPTP column. However, these putative phosphoproteins were not detected from parallel eluates collected from the column on which active SynPTP had been immobilized. Results suggest that these proteins could be the potential substrates for SynPTP from membrane fractions. Further study is required to identify these phosphoproteins.
Figure 3-23. Detection of potential substrates for SynPTP from membrane fractions of *Synechocystis* sp. PCC 6803. (A) Analysis of the proteins, eluted from active or inactive SynPTP that had been immobilized on a column, by SDS-PAGE on 12% (w/v) polyacrylamide gel, stained with Coomassie blue. Lanes 1, 3, 5, 7 contained proteins eluted from inactive SynPTP column with 50 mM, 100 mM, 150 mM, and 200 mM NaCl plus 50 mM phosphotyrosine respectively. Lanes 2, 4, 6, 8 contained proteins eluted from active SynPTP column with 50 mM, 100 mM, 150 mM, and 200 mM NaCl plus 50 mM phosphotyrosine respectively. (B) Western blot analysis of the corresponding gels. A monoclonal anti-phosphotyrosine antibody conjugated with horseradish peroxidase was used to detect phosphotyrosine containing proteins eluted from the active SynPTP column and mutagenically altered inactive
Identification of the Potential Substrates from Whole Cell Extracts of *Synechocystis* sp. PCC 6803:

Whole cell extracts of *Synechocystis* sp. PCC 6803 were prepared as described in Materials and Methods with the exception that cell lysis buffer included 0.1mM sodium orthovanadate, 0.1% Tween-20 (v/v), and 0.1% Triton-X-100 (v/v). A 7cm long IPG strip, pH 3–10, linear, was used for 2D gel electrophoresis. After completion of the second dimension, proteins were transferred to a PVDF membrane. The membrane was incubated either with or without 100 $\mu$g of active SynPTP in 10 mL buffer containing 20mM MES, pH 6.5, 2 mM DTT, 1mM EDTA and protease inhibitor cocktail (Sigma, St. Louis) overnight at 30°C. Following that, membranes were blocked for 1 hour at room temperature in TBST buffer containing 10 mM Tris HCl (pH 8), 150 mM of NaCl, 0.05% (v/v) Tween-20 and 1%(w/v) bovine serum albumin. Immunodetection was performed using anti-phosphotyrosine antibody, as described in Materials and Methods. Several phosphotyrosine containing proteins appeared on the immunoblot incubated without SynPTP. The presence of three phosphotyrosine containing proteins (indicated by arrowheads in Figure 3-25A) was not observed in the immunoblot incubating with SynPTP (Figure 3-25B). Results indicated that these three proteins were inactive for immunoreaction with antibody due to dephosphorylation by SynPTP and could be the potential substrates for SynPTP.

Two of these proteins (protein #1 and protein #2) were identified by mass spectral analysis, performed with a ThermoFinnigan LCQ DecaXP quadrupole
ion trap mass spectrometer. Peptide mass profiles were matched to the potential protein sources using NCBI/nr database through Mascot search developed by www.matrixscience.com. The results matched with the following proteins from *Synechocystis* sp. PCC 6803, which are:

1) Slr0737 (PsaD), Photosystem I subunit II, molecular weight 15.6 kDa, pI 8.9, and the GenBank accession no. 16329280. Mowse score for PsaD from proteome analysis was found 530. Predicted molecular mass and pI of this protein were matched with those of observed values.

2) Ssl3093 (CpcD), phycocyanin associated linker protein, molecular weight 9.3 kDa, pI 9.4, GenBank accession no. 16329820. Mowse score of this protein was found 37 from proteome analysis, while ions score > 38 indicated homology and ions score >54 indicated identity or extensive homology. However, the molecular mass and pI of this protein determined by 2D gel electrophoresis were matched with those of predicted values.

Third protein (Protein # 3) of the probable substrates was not identified since it was difficult to isolate the correct protein from the Coomassie blue stained acrylamide gel from the surrounding other proteins. Results of mass spectrometric analysis are summarized in Table 3-12. Observed pI values for PsaD and Ssl3093 were compared and found consistent with those values previously reported by Wang and Chitnis, 2000 (Table 3-13).
Figure 3-24. Identification of potential substrates for SynPTP from whole cell extracts of *Synechocystis* sp. PCC 6803. (A) Coomassie stained SDS-gel of 2D gel electrophoresis of whole cell extracts of *Synechocystis* sp. PCC 6803. (B) Immunoblot was incubated in the absence of active SynPTP. (C) Immunoblot was incubated in the presence of active SynPTP. Arrowheads in (B) indicate the positions of the potentials substrates which were immunoreactive with antibody in the absence of enzyme, but not following incubation in the presence of SynPTP (C).
Table 3-12. Proteomic analysis of PsaD and Ssl3093.

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>No. of peptides matched/total</th>
<th>Mowse Score</th>
<th>Predicted Mr</th>
<th>Observed Mr</th>
</tr>
</thead>
<tbody>
<tr>
<td>PsaD</td>
<td>22/481</td>
<td>530</td>
<td>15.6</td>
<td>16</td>
</tr>
<tr>
<td>Ssl3093</td>
<td>1/6</td>
<td>37</td>
<td>9.3</td>
<td>10</td>
</tr>
</tbody>
</table>

Predicted molecular mass was deduced from amino acid sequence, calculated by using PROTEIN CALCULATOR v3.2 program (http://www.scripps.edu/~cdputnam/protcalc.html). Observed molecular mass was determined from Coomassie blue stained acrylamide gel of 2D gel electrophoresis.

PsaD

1 MTELSGQPPK FGGSTGGLLS KANREEKYAI TWTSASEQVF EMPTGGAAIM
51 NEGENLLYLA RKEQCLALGT QLRTKFKPKI QDYKIYRVYP SGEVQYLHPA
101 DGVFPEKVNE GREAGGTKTR RIGQNPEPVT IKFSKGAPYE V

Ssl3093

1 MLGQSSLVGY SNTQAANRVF VYEVSGLRQT DANENSAHDI RRSGSVFIKV
51 PYARMNDEMR RISRLGGTIV NIRPYQADSN EQN

Figure 3-25. Matched peptides of PsaD and Ssl3093 were shown in bold red.
Table 3-13. Comparison of deduced pI and observed pI of PsaD and Ssl3093

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>Deduced pI</th>
<th>Observed pI</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PsaD</td>
<td>8.9</td>
<td>9.5</td>
<td>This report</td>
</tr>
<tr>
<td>PsaD</td>
<td>8.95</td>
<td>9.6, 9.5, 9.876, 7.06</td>
<td>Wang and Chitnis, 2000</td>
</tr>
<tr>
<td>Ssl3093</td>
<td>9.4</td>
<td>9.5</td>
<td>This report</td>
</tr>
<tr>
<td>Ssl3093</td>
<td>9.39</td>
<td>9.6</td>
<td>Wang and Chitnis, 2000</td>
</tr>
</tbody>
</table>

Deduced pI was determined from amino acid sequence, calculated by using PROTEIN CALCULATOR v3.2 program (http://www.scripps.edu/~cdputnam/protcalc.html).

Observed pI was determined from Coomassie blue stained acrylamide gel of 2D gel electrophoresis.
Summary of Specific Aim-3:

A review of all *in vitro* experiments indicate that probable substrates of SynPTP include: i) Sll1577, phycocyanin β subunit; ii) Sll1578, phycocyanin α subunit; iii) PsaD, photosystem subunit II; iv) Ssl3093, phycocyanin associated linker protein.

Focusing on phycocyanin α and β, we further examined whether these two substrates are phosphorylated *in vivo*. After purification of phycocyanin from soluble fractions of *Synechocystis* sp. PCC 6803 cell extracts, by anion exchange or size exclusion chromatography, it was observed that the purified fractions of phycocyanin were immunoreactive with an anti-phosphotyrosine antibody. After incubation with active SynPTP, it was observed that phycocyanin no longer immunoreacted with anti-phosphotyrosine antibody. The predicted pI of phycocyanin matched with observed pI, examined by 2D gel electrophoresis. Different mutant strains of *Synechocystis* sp. PCC 6803 lacking phycocyanin, PAL and CK mutants, displayed the low level of immunoreactivity of antibody toward phycocyanin in comparison with the wild type strain. The whole cell extracts of *Synechocystis* sp. PCC 6803 cells, grown under nitrogen or phosphate starvation, revealed weaker immunoreactivity of antibody toward phycocyanin phosphorylation than the whole cell extracts of wild type strain.
Chapter IV

Discussion:

Specific Aim-1: To identify potential protein tyrosine phosphatase from *Synechocystis* sp. PCC 6803.

Phosphatase Activity of Potential Protein Product of *slr0328*, SynPTP:

Mutagenically altered Cys7Ser recombinant SynPTP failed to dephosphorylate the RCML (\(^{32}\)P-Tyr), casein (\(^{32}\)P-Tyr) or pNPP in detectable amounts, indicating that recombinant SynPTP expressed in *E. coli* is the source of phosphatase activity (Table 3-1). Recombinant SynPTP with a C-terminal His tag and recombinant SynPTP without any fusion tag exhibited similar levels of phosphatase activity toward casein (\(^{32}\)P-Tyr) or pNPP (Table 3-1). Recombinant SynPTP with an N-terminal His tag, on the other hand, displayed much lower catalytic activity than the other two recombinant proteins toward RCML (\(^{32}\)P-Tyr) or pNPP, which suggested that the N-terminal His tag effects the active site and/or folding of the protein.

Kinetic Parameters of SynPTP:

Kinetic analysis of the activity of SynPTP toward pNPP indicated that its \(K_m\) and \(V_{\text{max}}\) values are comparable with those of other known bacterial low molecular weight PTPs (Table 3-2). It was observed that \(V_{\text{max}}\) value of SynPTP is almost same with that of yeast PTP Ltp1 and 4-fold lower than that of another yeast PTP, Stp1. However, the \(V_{\text{max}}\) value of SynPTP is ~35 fold lower than the mammalian PTP, BHPTP. Results suggested that SynPTP displays similar
pattern of its catalytic function toward pNPP with that of other known bacterial LMW PTPs.

Specific Aim-2: To determine whether SynPTP can specifically hydrolyze phosphomonoesters from tyrosine residues on protein substrates as a PTP or hydrolyze both tyrosine and serine/threonine residues on protein substrates as a DSP.

We utilized three strategies to determine substrate specificity of SynPTP.

(i) Comparison of the Catalytic Activity of SynPTP toward α-Napthyl Phosphate and β-Napthyl Phosphate:

Napthyl phosphate isomers were particularly used since they represent as “diagnostic” substrates to distinguish between PTPs and DSPs in vitro (Figure 3-6). From our results (Table 3-3) it was observed that SynPTP was catalytically active toward β-napthyl phosphate but not toward α-napthyl phosphate, which indicated that the geometry of the substrate binding pocket of SynPTP is similar to that of a protein tyrosine phosphatase.

(ii) Examination of the Catalytic Activity of SynPTP toward Exogenous Tyrosyl or Seryl Phosphorylated Protein Substrates:

To further examine whether SynPTP functions catalytically as a DSP or PTP, we used three exogenous protein substrates, casein, MBP, and RCML, and these substrates were phosphorylated either on tyrosine residues or seryl
residues. Our results indicated (Table 3-5) that SynPTP displayed catalytic activity toward tyrosyl phosphorylated all three protein substrates but failed to dephosphorylate the same seryl phosphorylated substrates. Results suggested that SynPTP exhibited its catalytic function similar to a PTP not as a DSP.

(iii) Determination of the Effects of Known Phosphatase Inhibitors on Catalytic Activity of SynPTP:

We further used several known inhibitors for protein phosphatases to examine the influence of these inhibitors on phosphatase activity of SynPTP (Table 3-7). Our data indicated that the catalytic activity of SynPTP was totally blocked by ammonium molybdate or zinc sulphate (Zn$^{+2}$), which are potent inhibitors for PTPs. Since sulphate (SO$_4^{2-}$) might mimic phosphate (PO$_4^{3-}$), we examined effect of sodium sulphate on the catalytic activity of SynPTP. It was observed that sodium sulphate had no inhibitory effect on phosphatase activity of SynPTP, which confirmed that Zn$^{+2}$ behaves as a potent inhibitor for SynPTP. The phosphatase activity of SynPTP was inhibited when sodium orthovanadate, a known inhibitor of PTPs, was used. It was also noticed that there was no inhibitory effect for phosphatase activity of SynPTP with sodium fluoride and okadaic acid, which are inhibitors of many Ser/Thr phosphatases. Results suggested that the sensitivity of the catalytic function of SynPTP for these inhibitors followed the similar pattern with other known PTPs (Soulat et al., 2002; Koul et al., 2000; Grangeasse et al., 1998; Li and Strohl, 1996; Tonks et al., 1988). Taken together, the results indicate that SynPTP is a PTP and not a DSP.
Specific Aim-3: To identify potential physiological substrates for SynPTP in *Synechocystis* sp. PCC 6803:

**Tyrosine Phosphorylation of Phycocyanin:**

To identify physiological substrates for SynPTP, we performed an *in vitro* trapping experiment using active recombinant SynPTP or mutagenically altered C7SD125A SynPTP as a ligand. Our data suggested (Figure 3-10) that two proteins, 19 kDa and 17 kDa, were probable substrates for SynPTP. These were identified by mass spectral analysis as the α and β subunits of phycocyanin (Table 3-8).

For many years, the issue whether phycocyanin is indeed phosphorylated has been controversial, even though this phosphorylation event was reported several times in the past (Sanders et al., 1989; Harrison et al., 1991; Mann and Newman, 1999). Piven and co-workers (Piven et al., 2005) ruled out the possibility of phosphorylation of phycocyanin using phosphoprotein staining gel dye Pro-Q Diamond or phospho-threonine antisera for their experimental analysis. Their data suggested that the signals obtained for phycocyanin from Pro-Q Diamond staining arose due to its self-fluorescence not for phycocyanin phosphorylation. They concluded from their analyses that the "chemiluminescence" signals for phycocyanin originated from immunological analysis using phospho-threonine antisera were due to its self-fluorescence, not for phosphorylation.
Therefore, we examined whether the "chemiluminescence" signals observed when Western blots treated with an anti-phosphotyrosine antibody, was due to tyrosine phosphorylation of phycocyanin or its intrinsic autofluorescence. Our results suggest (Figure 3-16) that phycocyanin is tyrosine phosphorylated, since signal was observed only following the incubation with antibody. Next, we examined the specificity with which the anti-phosphotyrosine antibody recognized the epitope of a phosphotyrosine on phycocyanin. The antibody (RC20, Amersham Biosciences) we used was a monoclonal and conjugated to the reporter enzyme horseradish peroxidase. Therefore, no secondary antibody was used. This was important since many commercially available secondary antibodies immunoreact with bacterial proteins (McCartney et al., 1997; BD Biosciences, CA; Smith et al., 2001). It was observed (Figure 3-17) that immunoreactivity was abolished by the addition of free phosphotyrosine, but not free phosphoserine or free tyrosine. These data indicate that the antibody was able to specifically recognize the epitope for phosphotyrosine.

Next, we examined the immunoreactivity of purified phycocyanin. Phycocyanin was isolated from the soluble fraction of *Synechocystis* sp. PCC 6803 by anion exchange or size exclusion column chromatography. The purified fractions immunoreacted with the RC20 antibody, and two phosphotyrosine proteins appeared on immunoblots at the expected molecular size range for phycocyanin $\alpha$ and $\beta$, 15 kDa to 20 kDa (Figure 3-12 and Figure 3-14). Mass spectral analysis of these two proteins (indicated by arrowhead in Figure 3-12) revealed them to be $\alpha$ and $\beta$ subunits of phycocyanin. Multiple immunoreactive
protein spots were observed in the immunoblot following 2D gel electrophoresis (Figure 3-13) in a pI range of 4.5 to 5.5 and molecular size range of 15 kDa to 20 kDa, expected for phycocyanin α and β subunits. Results indicated that the pI ranges of phycocyanin α and β subunits were consistent with those values of phycocyanin previously reported by Wang and Chitnis, 2000 (Table 3-10). The presence of multiple protein spots for phycocyanin in the immunoblot (Figure 3-13B) could possibly be due to post-translational modifications.

Immunoreactivity of antibody for phycocyanin was abolished when a portion of these purified fractions was incubated with active recombinant SynPTP (Figure 3-18). The results indicated that SynPTP was able to dephosphorylate tyrosyl phosphorylated phycocyanin. It was observed that immunoreactivity of antibody toward phycocyanin was much lower in the CK and PAL (lacking of phycocyanin) mutant strains of *Synechocystis* sp. PCC 6803, as compared to wild type (Figure 3-20, 3-22). This would be expected if phosphotyrosyl phycocyanin was the source of signal for immunoreactivity. A similar effect on the apparent immunoreactivity for antibody toward phycocyanin was observed when cells were deprived of nitrogen or phosphate (Figure 3-22). Since phycocyanin is degraded when cells are deprived of nutrient, it was expected that there would be little or no immunoreactivity of antibody. Our data suggest that phycocyanin is tyrosyl phosphorylated in the wild type strain.

Based on above observation and reviewing our all results, we propose that α and β subunits of phycocyanin are a) tyrosine phosphorylated and b) are substrates for SynPTP.
Possible Function of the Phosphorylation of Phycocyanin:

In green plants, phosphorylation of proteins in the light harvesting complex II (LHCII) is known to regulate the distribution of absorbed excitation energy between photosystem I (PSI) and photosystem II (PSII) such that light-limited photosystem receives more energy while the light-saturated photosystem receives less (Bennett et al., 1980; Allen et al., 1981; Allen, 1983; Forsberg and Allen, 2001). Energy transfer to PSI is favourable under PSII light (650 nm) whereas energy transfer to PSII is preferred under PSI light (730 nm) (Forsberg and Allen, 2001). The state of adjustment to PSI light is known as state 1 and the state of adjustment to PSII light is known as state 2. The transition between two states is described as light-state transition (Allen and Nilsson, 1997).

It was reported that phosphorylation of LHCII alters its three-dimensional structure (Anderson et al., 1982; Allen and Nilsson, 1997; Nilsson et al., 1997). This conformational change induces the dissociation of the phospho-LHCII trimer into monomeric phospho-LHCII. The monomers are free to migrate from PSII to PSI (Barber et al., 1982; Bennet, 1983; Howarth, et al., 1982). After dephosphorylation, unphosphorylated LHCII monomers may trimerize at the periphery of PSII. Phosphorylation of LHCII leads the adjustment of the stoichiometry of two photosystems (Anderson et al., 1982; Allen and Nilsson et al., 1997). Figure 4-1 shows a model explaining the possible role of phospho-LHCII in pea (*Pisum sativum*).

In cyanobacteria, phycobilisomes are a light harvesting complex composed of various biliproteins and linker polypeptides. Phycocyanin is one of
the major biliproteins and functions as a light-harvesting antenna for photosynthetic reaction centers. We propose that phosphorylation of phycocyanin in cyanobacterium *Synechocystis* sp. PCC 6803 might be involved in the regulation of excitation energy distribution in a similar way as occurs for LHCII in higher plants. At the state of adjustment to PSII light (state 2) phosphorylation of phycocyanin might induce dissociation of phosphorylated phycobilisome trimers to monomeric phosphorylated phycobilisomes. The dissociation of phosphorylated phycobilisomes may induce lateral movement of monomeric phosphorylated phycobilisomes to bind preferentially to PSI at the state of adjustment to PSI light (state 2). After dephosphorylation monomeric unphosphorylated phycobilisomes may assemble in trimer form and reassociate with PSI at state 2 light adaptation.

In *Synechocystis* sp. PCC 6803, the ORFs next to slr0328 encode a potential glucokinase (slr0329) and a deduced iron transport protein (slr0327). But it was observed that in *Prochlorococcus marinus* MIT9313, *Prochlorococcus marinus* MED4, and *Synechococcus* sp. WH8102, ORFs encoding potential low molecular weight PTPs are present adjacent to the ORFs encoding potential phycoerythrobilin:ferredoxin oxidoreductase or phycocyanobilin lyase (*pmt1688, pmt1687* from *Prochlorococcus marinus* MIT9313; *pmm1591, pmm1592* from *Prochlorococcus marinus* MED4, and *synw2026, synw2025* *Synechococcus* sp. WH8102). In *Synechococcus* sp. WH8020, ORF65, encoding a potential low molecular weight PTP, is present next to the ORF encoding phycoerythrin (Wilbanks and Glazer, 1993). In cyanobacteria, phycocyanobilin:ferredoxin
oxidoreductase is involved in the electron transfer from ferredoxin to tetrapyrrole to produce phycocyanobilins (Hagiwara, et al., 2006). Phycocyanobilin lyase regulates the degradation of phycobilisome complex through phosphorylation or dephosphorylation of phycobilisomes (Dolganov and Grossman, 1999). This information suggests that these proteins may regulate the function of phycobilisomes in their corresponding organisms. Since, it was observed that the ORF encoding potential LMW PTP is present next to the ORF encoding either potential phycocyanobilin lyase or phycocyanobilin:ferredoxin oxidoreductase in these organisms mentioned above, therefore it could be possible that the potential LMW PTP may involve in the regulation of the function of phycobilisomes, hence photosynthesis, in their respective organisms. Thus the above information strengthens my proposal that SynPTP in Synechocystis sp. PCC 6803 may be involved in the regulation of photosynthesis.
Figure 4-1: A schematic diagram for possible explanation of role of phospho-LHCII in *Pisum sativum* (adapted from Foresberg and Allen, 2001).

**Phosphorylation of PsaD:** Our preliminary data from whole cell extracts of *Synechocystis* sp. PCC6803 implied that PsaD is tyrosyl phosphorylated and a possible substrate for SynPTP *in vitro* (Figure 3-24). The presence of this protein was observed in the immunoblot incubated without SynPTP, but not in the immunoblot incubated with SynPTP. Results indicated that Psad was dephosphorylated by SynPTP and therefore not detected by anti-
phosphotyrosine antibody, which makes this protein as a possible substrate for this phosphatase. Further study is required to establish whether PsaD is subject to tyrosyl phosphorylation in *Synechocystis* sp. PCC 6803 and confirm whether it is substrate for SynPTP.

**Potential Function of the Phosphorylation of PsaD:**

Detailed studies of PsaD in *Synechocystis* sp. PCC 6803 suggested that this photosystem I subunit plays multiple functions. It stabilizes the complex by binding to other subunits (A, B, C, L) and forming the PSI trimer. PsaD is the major docking site for ferredoxin (fd) (Chitnis and Chitnis, 1993; Kruip et al. 1993; Xu, et al., 1994; Armburst et al., 1996; Hanley, et al., 1996; Kruip et al., 1997; Bottin et al., 2001; Lagoutte, et al., 2001). Mutagenic alteration of PsaD led to an impairment of cell growth at temperatures above 30°C (Lagoutte, et al., 2001). Phosphorylation of PsaD could possibly stabilize or destabilize the PSI complex or might change the ferredoxin docking site. Further study is required to determine how phosphorylation of PsaD effects these functions.

**Linker Protein Phosphorylation:** A phycocyanin-associated rod linker protein, Ssl3093, of ~9.5 kDa was detected from whole cell extracts of *Synechocystis* sp. PCC 6803 (Figure 23) as a possible substrate for SynPTP *in vitro*. The presence of this protein was detected in the immunoblot incubated without SynPTP, but not in the immunoblot incubated with SynPTP, suggesting that Ssl3093 was dephosphorylated by SynPTP. Phosphorylation of linker proteins (33 kDa, 35
kDa, 99 kDa, 27 kDa) on threonine in *Synechocystis* sp. PCC 6803 was reported previously by Piven et al., (2005). Future studies need to be performed to firmly establish tyrosine phosphorylation of this linker protein. Rod linker proteins stabilize the structure of phycobilisome complex. It was proposed by Piven et al., (2005) that the dephosphorylation of linker proteins may function as a signal for protein degradation when disassembly of the phycobilisome complex has started, while phosphorylation may occur before assembly of phycobilisome complex.

**Mass Spectral Analysis versus Immunological Analysis:**

We identified the potential tyrosyl phosphorylated proteins from either soluble fractions or whole cell extracts of *Synechocystis* sp. PCC 6803 as listed above. However, no phosphopeptides of these proteins were observed during mass spectral analysis. Possible reasons for this could be:

i) The stoichiometry of tyrosyl-phosphorylated proteins might be very low, therefore it could be difficult to analyze the low amount of phosphopeptides in mass spectrometric analysis; ii) Phosphopeptides tend to be negatively charged (Mann et al., 2002; Reinders and Sickmann, 2005). Generally electrospray mass spectrometry is performed in the positive mode, therefore the signals for phosphopeptides may not observed as intense peaks and may be masked by the signals for other peptides (McLachlin and Chait, 2001; Reinders and Sickmann, 2005); iii) Since phosphopeptides are hydrophilic, they do not bind well to the columns which are generally used for purification of peptides (C18 tips) prior to mass spectrometry. This might cause the loss of some phosphopeptides (Mann
et al., 2002). Overall, analysis of phosphopeptides in mass spectometry is still a challenging task.

On the other hand immunological analysis, using an anti-phosphotyrosine antibody, is a common and established tool. From dot blot assay, it was observed that the presence of 10 femtomole of phosphotyrosine protein in an immunoblot is enough to detect that protein (data not shown). Minimum amount of pure phosphotyrosine peptides required for mass spectral analysis, is 100 picomole (information was obtained from Michigan Proteome Consortium). Therefore, immunological assay is a much more sensitive method than mass spectral analysis.

We relied on Immunological analysis to support our experimental data on the basis of: i) We examined whether the anti-phosphotyrosine antibody, used for all immunodetection experiments to detect the tyrosyl phosphorylated proteins, was specific to bind a phosphotyrosine epitope of a phosphotyrosine protein. Our results suggested (Figure 3-17) that the antibody was able to recognize specifically the phosphotyrosine epitope and outcompeted with free phosphotyrosine but not with free phosphoserine or free tyrosine; ii) *Synechocystis* sp. PCC 6803 cells were grown in low light intensity, 10 \( \mu \text{mol} \text{ photons m}^{-2} \text{ s}^{-1} \). Stoichiometry of tyrosyl-phosphorylated proteins could be very low in this condition. Therefore, this low amount of tyrosyl-phosphorylated protein could be sufficient to be detected by Western blot analysis, but not by mass spectral analysis; iii) These potential tyrosyl-phosphorylated proteins were incubated with SynPTP or without SynPTP and immunodetections were
performed. Results indicated that SynPTP was able to dephosphorylate these putative tyrosyl phosphorylated proteins in vitro, suggesting that these proteins are possible substrates for SynPTP (Figure 3-18 for phycocyanin; Figure 3-24 for PsaD and Ssl3093); iv) The predicted molecular mass and pI of these potential tyrosyl phosphorylated proteins were matched with the observed mass and pI from Coomassie blue stained acrylamide gel (Table 3-10; 3-11).

However, there could be a possibility that the immunologically reactive species is a trace contaminant, and not these proteins listed above. Further studies need to be performed to solve this problem. This possible contamination could be eliminated if these potential tyrosyl-phosphotylated proteins can be overexpressed inside the cells with a fusion tag. Presence of fusion tag in the recombinant protein of interest could distinguish it from the native protein inside the cells by the difference of molecular size. After isolating the recombinant protein through fusion tag, immunodetections would be performed to detect phosphotyrosine protein and thus the possible contamination could be eliminated.
Conclusions

A homology search of protein tyrosine phosphatases in *Synechocystis* sp. PCC 6803 revealed that the potential protein product of *slr0328*, named SynPTP, shares the common signature motif CX5RS/T and conserved Asp with known low molecular weight protein tyrosine phosphatases. Kinetic analysis suggested that $K_m$ and $V_{\text{max}}$ values of SynPTP are comparable with other known bacterial low molecular weight PTPs and yeast PTP (Ltp1). Results indicated that SynPTP is specific toward phosphotyrosyl protein substrates. Results also suggested that SynPTP is able to dephosphorylate β-napthyl phosphate but not α-napthyl phosphate. The catalytic activity of SynPTP is inhibited by many known inhibitors of PTPs, but not by inhibitors of many Ser/Thr phosphatases. Reviewing these results we conclude that SynPTP is a member of low molecular weight PTP superfamily.

Two tyrosine phosphorylated proteins, the α and β subunits of phycocyanin, were identified as the substrates for SynPTP from soluble fractions of *Synechocystis* sp. PCC 6803. Preliminary results indicated that PsaD, a subunit of PSI, and Ssl3093, a phycocyanin-associated rod linker protein, were potential substrates of SynPTP. These proteins were detected from whole cell extracts of *Synechocystis* sp. PCC 6803. Taken together, it is speculated that SynPTP may be involved in the regulation of photosynthesis.
Future Directions

Further studies are needed to identify the phosphorylation sites on phycocyanin, PsaD, and Ssl3093. This could be performed by i) mass spectral analysis, ii) mutagenic alteration of each tyrosine residue (one residue at a time) in these proteins, expressing those mutagenically altered proteins inside the cells with fusion tag, isolating these proteins through fusion tag, and performing immunodetection for phosphotyrosine proteins. Future work needs to be performed to identify those proteins, which were detected as potential substrates for SynPTP from membrane fractions of *Synechocystis* sp. PCC 6803 (Figure 22). Further studies need to be conducted to establish the tyrosyl phosphorylation of PsaD and Ssl3093 as it was performed for phycocyanin (several lines of evidences).
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