CHAPTER IV

DISCUSSION

Since phosphate is most often the limiting nutrient in the freshwater ecosystem, the two-component system involved in phosphate sensing was studied extensively in *E.coli* and also in cyanobacteria including *Synechococcus* PCC 7942 (Aiba et al., 1993), and *Synechococcus* WH 7803 (Watson et al., 1996). The histidine kinase and response regulator pair Sll0337-Slr0081 are involved in this response to phosphate-limiting conditions in *Synechocystis* sp. PCC 6803 (Hirani et al., 2001) therefore, it is of interest to study the structural component of Sll0337 and Slr0081 involved in this regulation by using site-directed mutagenesis.

Site-directed mutagenesis requires the known sequence of the region of the target gene (Thiel, 1994). The sequences of Sll0337 and Slr0081 were obtained from Cyanobase. We found that the sequence of Sll0337 from cyanobase differed from that obtained from the amino acid alignment of PhoR. On the other hand, there was no difference in the alignment or annotation of Slr0081. The Sll0337 protein predicted from Cyanobase lacked 47 amino acids (Fig. 7). Therefore the start site of *sll0337* was investigated by RT-PCR and 5'-RACE-PCR. From the sequence of the PCR product, there were 38 nucleotides located upstream of ATG (Fig. 9). If the GTG were the start codon, there would be 179 nucleotides upstream of the *sll0337* gene. Since 5'-UTR of cyanobacterial expected to lie about 50-100 bp upstream of translation initiation site (Curtis and Martin, 1994), this result indicates that ATG rather than GTG should be the start codon of *sll0337*. After the ORF of the *sll0337* and *slr0081* were obtained,

the function of specific amino acids of PhoR and PhoB in *Synechocystis* sp. PCC 6803 were examined by site-directed mutagenesis.

Site-directed mutagenesis, causing one or more specific changes in nucleotide sequence, has been used primarily in unicellular cyanobacteria for the study of photosynthesis. The plasmid with the mutation is introduced into the wild type cyanobacterium by gene transfer. The mutagenesis plasmid is constructed by insertion an antibiotic resistance gene near but outside of the coding region of the interested gene. Selection for antibiotic resistance typically produces mutants. However, in some cases recombination events occur at only the segment of DNA between the mutation and the antibiotic resistance cassette gene. There will produce antibiotic-resistant strains that are not mutated at the site of interest. Therefore initially a cyanobacterial strain with a deletion of the interested gene is created. Using deletion mutants when the plasmid with the site-directed mutation is transferred to the cyanobacterial deletion strain, antibiotic-resistant recombinations can be formed only by crossover events outside the interested gene. Thus all such recombinants should contain the site-directed mutation within that gene (Thiel, 1994).

The selectable markers in oligonucleotide-directed mutagenesis are antibiotic-resistance cassettes. The photoautotrophic growth was followed to confirm that antibiotic cassettes, included in the mutagenesis and deletion plasmid constructs had no unexpected deleterious effect in the resulting strains. The deletion strain and control strains showed similar growth to wild type in both normal and phosphate-limiting BG-11 (Fig. 14 and 15). The results suggested that the antibiotic-resistance cassette had no effect on the phenotype of the mutant strains.

Previously, a Thr-214 to Asn substitution in Sll0337 resulted in up-regulation of

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alkaline phosphatase activity in phosphate-sufficient conditions. However, removal of the negative regulator PhoU in this mutant reversed this effect even though the ΔPhoU strain exhibited constitutive up-regulation of alkaline phosphatase activity. The results can suggested that Thr-214 may interact with PhoU (Simpson, 2003). Moreover, the Asp-88 is predicted to be the phosphorylation site in the Slr0081. In this study, the Thr-214 of Sll0337 and Asp-88 of Slr0081 were the target of specific amino acid mutation.

The amino acid residue corresponding to the Thr-214 of the histidine kinase from *E. coli* contains both polar and nonpolar residues such as Ser, Thr, Asn, Ala and Gly (Stock et al., 1995). The position 214 of histidine kinase from *Synechocystis* sp. PCC 6803 can be Thr, Asn and Ala therefore the T214N has been previously constructed (Hirani, 2001). In this study the Thr-214 was further replaced with Ser, Ala, Arg, Gln, Gly and compared to the T214N and wild-type strains. The alkaline phosphatase activity of the T214R mutant was not induced (Fig. 17, lane 7) whereas other Thr-214 mutations produced strains that expressed alkaline phosphatase activity (Fig. 17, lane 2-6) under phosphate-limiting condition. The identification of Thr-247 in the H box on the helix I of the dimerization histidine phosphotransfer domain of EnvZ, indicated that the charge of the environment at the active site of a histidine kinase may play a role in shifting the reaction equilibrium in favor of reverse phosphotransfer of the phosphoryl group from OmpR to EnvZ (Dutta et al., 2000). The Thr-247 corresponds to the Thr-211 in Sll0337. However, the Thr-214 is still in the active site so the substitution of Thr-214 with the positively charged residue Arg may be involved in the reverse phosphotransfer from phosphorylated Slr0081. Moreover, Thr-214 corresponds to Thr-250 in EnvZ and this Thr-250 participates in

the formation of helix I in close proximity to the phosphorylated His-243 in H motif of EnvZ (Qin et al., 2003; Tomomori et al., 1999). It is possible that replacing Thr-214 with Arg of Sll0337 may be related to the charge repulsion between the histidine and arginine. This repulsion could disrupt the PhoR function in *Synechocystis* sp. PCC 6803.

Mutant strain T214S, T214A, T214Q, T214G and T214N exhibited a very interesting result (Fig. 17). These mutants displayed constitutive induction of alkaline phosphatase activity. However, the activity of alkaline phosphatase in normal BG-11 was not as high as that observed in phosphate-limiting conditions. These results indicated that the mutation in the T214S, T214A, T214Q, T214G and T214N strains leads to an insufficient repression of the *pho* regulon under phosphate-sufficient condition while retaining the inducing activity under phosphate-limiting conditions. The residue Thr-214 corresponds to Thr-220 in the PhoR from *E. coli*. The T220N mutant was found to be defective in the repression of the *pho* regulon in an environment of excess phosphate (Wanner, 1987; Yamada et al., 1989). Since the T220N mutant was insensitive to the environmental phosphate concentration, the Thr-220 residue may be important for receiving the environmental signal and therefore this mutant is considered to constitutively activate PhoB.

The repression of the *pho* regulon is attributed to the PhoU protein. Moreover, this protein may interact with PhoR to form a repression complex (Wanner, 1995). To investigate whether Thr-214 interacts with PhoU, The Thr-214 mutants in the absence of PhoU were constructed. The role of the *phoU* gene product in the regulation of alkaline phosphatase synthesis was originally inferred from the phenotype of the *phoU*35 mutation. This mutation causes constitutive synthesis of alkaline phosphatase

in *E. coli* (Nakata et al., 1984). Furthermore, the alkaline phosphatase activity was highly induced when the *phoU*35 null mutants were grown under phosphate-sufficient condition. The result indicated that PhoU is a negative regulator of alkaline phosphatase. In the present study, the ΔPhoU mutant from *Synechocystis* sp. PCC 6803 gave a similar result to that *phoU*35 null mutant confirming that PhoU is a negative regulator of the phosphate-sensing pathway under phosphate sufficient conditions.

When the Thr-214 mutants with the *phoU* interruption were obtained, the alkaline phosphatase activity was compared with that of the ΔPhoU and wild type strains. The results from all mutants showed the same trend as that of ΔPhoU mutant (Fig. 21, lane 2-6) except for T214N:ΔPhoU (Fig. 21, lane 7) and T214R:ΔPhoU (Fig. 21, lane 8). There was no induction of the alkaline phosphatase activity of T214R:ΔPhoU. This result confirmed that the substitution of Thr-214 with Arg affects the function of PhoR resulting in the disruption of signal transduction. Even when PhoU was removed, the *pho* regulon remained non-functional. The mutant strain T214N:ΔPhoU was shown to up-regulate alkaline phosphatase activity only marginally under both phosphate-sufficient and phosphate-limiting conditions (Fig. 21 lane 7). The residue Thr-214 corresponds to Thr-250 of EnvZ in *E. coli*. The T250C was constructed in EnvZ. It seems that T250C did not disrupt the four-helix-bundle structure of the dimerization domain EnvZ (Qin et al., 2003). Therefore the ability to form histidine kinase dimers may still be retained in the T214N mutant. In the absence of PhoU, with insufficient ability to phosphorylate Slr0081, the alkaline phosphatase activity was poorly induced under phosphate-limiting condition. The result suggested

that PhoU may interact with Thr-214 in the helix I of Sll0337 possibly through the dimer formation.

The substitution of aspartate at position 55, which is the site of phosphorylation, with glutamine or alanine renders OmpR unable to activate transcription (Walthers et al., 2003; Kanamaru et al., 1990). The Asp-55 corresponds to Asp-88 in Slr0081. The D88N mutant had no induction of alkaline phosphatase activity (Fig. 24, lane 2). The result indicated that Asp-88 should be the phosphorylation site of Slr0081 and this mutation affected the *pho* regulon expression. There was no induction of the alkaline phosphatase activity in D88N:ΔPhoU cells (Fig. 28, lane 3). This result confirmed that the substitution of Asp-88 with Arg affects the function of PhoB resulting in the disruption of the signal transduction. Even when the PhoU was removed, the *pho* regulon remained non-functional.

Cyanobacterial cells deficient for any of a number of different nutrients exhibit a decline in cellular pigmentation known as chlorosis or bleaching. In some cases chlorosis involves the degradation of pigments. In the other cases, there is no active degradation, but the levels of the different pigments per cell decline because cell division continues after pigment synthesis stops (Grossman et al., 1994). Under phosphate-limiting conditions, the *Synechocystis* sp. PCC 6803 cells become pale yellow-green instead of the blue green colour observed in the presence of sufficient phosphate. This bleaching was confirmed by absorption spectra in Figs. 18, 22 and 25 showing that the the absorption spectra of mutant cells in normal BG-11 showed the maximum absorption at 680 and 440 nm, due to chlorophyll *a*, and the peak at 620 nm, corresponding to phycobilins. However, under phosphate-limiting conditions these are significantly reduced. In *Synechococcus* sp. PCC 7942, during nutrient limitation,

when the anabolism of the cell is slowed down, $NADP^+$, the final electron acceptor of the photosynthetic electron transport chain, is not recycled as fast as under nutrient-replete conditions and the electron carriers are maintained in a reduced state. Retention of the phycobillisomes and light absorption during nutrient-limited growth could further reduce the electron carriers and result in photodamage. Therefore, the degradation of phycobillisomes might help minimize the absorption of excess excitation energy (Schwarz and Grossman, 2001). It can be explained that the reduction of the chlorophyll *a* and phycobilins may also help protection of the *Synechocystis* sp. PCC 6803 cells from phototoxicity under phosphate-limiting conditions.

In *Escherichai coli*, there are some reports about the cross regulation between endogenous acetyl phosphate and the response regulator PhoB in the absence of PhoR, *in vivo* (Kim et al., 1996). The AckA mutation leads to elevated acetyl phosphate levels because of a block in its breakdown. The Pta mutation leads to lower acetyl phosphate levels because of a block in its synthesis (Wanner, 1992). Acetate kinase and phosphotransacetylase are encoded by *sll1299* and *slr2132*, respectively. Therefore, the ΔSll1299/ΔSll0337, ΔSlr2132/ΔSll0337 and ΔSll1299/ΔSlr2132 /ΔSll0337 mutants in *Synechocystis* sp. PCC 6803 were created to examine this cross regulation. The ΔSlr2132/ΔSll0337 and ΔSll1299/ΔSlr2132/ΔSll0337 mutant produce low level of acetyl phosphate so that it cannot phosphorylate Slr0081. Therefore the alkaline phosphatase activity of these strains should not be induced. Fig. 41 showed the expected results (Fig. 41, lane 3 and 4). If the Slr0081 can be phosphorylated by endogenous acetyl phosphate, the alkaline phosphatase activity should be induced

under phosphate-limiting conditions. However, the alkaline phosphatase activity of ΔSll1299/ΔSll0337 was not observed in both normal and phosphate-limiting BG-11 (Fig. 41, lane 2). The result suggested that Slr0081 may not be cross regulated by endogenous acetyl phosphate in *Synechocystis* sp. PCC 6803.

Previously, the supplementation of acetyl phosphate in phosphate-limiting BG-11 was shown to restore endogenous ATP because the cells grown under this condition showed similar growth to that of the wild type in normal BG-11 (Hirani, 2001). However, it is not known if acetyl phosphate is transported directly into the cells or of it is hydrolysed (Hirani, 2001). The ΔSll1299/ΔSlr2132/ΔSll0337 strain was used to examine the ATP production from exogenous acetyl phosphate. Although most cyanobacteria appear unable to take up organic phosphate containing molecules, the production of periplasmic alkaline phosphatase under phosphate-limiting condition allows the cells to scavenge phosphate available through the hydrolysis of a wide variety of organic phosphate sources. The usable phosphate is transported across cytoplasmic membrane by phosphate transporter systems (Grossman et al., 1994). The alkaline phosphatase activity was abolished in the Sll0337 mutant resulting in no alkaline phosphatase in the ΔSll1299/ΔSlr2132/ΔSll0337 strain. However, the acetyl phosphate was still able to restore endogenous ATP because this strain could grow under phosphate-limiting BG-11 containing acetyl phosphate despite the absence of alkaline phosphatase induction (Fig. 42). This result suggested that the acetyl phosphate is transported directly into the cells.

Moreover, if transported acetyl phosphate produced the endogenous ATP via ack and pta pathway, this mutant strain would not be able to survive because there was

no acetate kinase and phosphotransacetylase. Our results showed that this strain could still grow under phosphate-limiting BG-11 containing acetyl phosphate (Fig. 42). The result suggested that the ack and pta pathways are not involved in ATP production from exogenous acetyl phosphate. The acetyl phosphate may provide phosphate group via a different reaction resulting in the hydrolyzes of acetyl phosphate.

From Cyanobase, we found that acetate kinase and phosphotansacetylase of *Synechocystis* sp. PCC 6803 were encoded by *sll1299* and *slr2132* genes, respectively. Therefore these genes were selected and investigated for their mRNA expression under phosphate starvation. Transcriptional analysis of gene clusters has traditionally been carried out by Northern blotting. However the reverse transcription RT-PCR has previously been used to detect low levels of mRNA transcript in the polyketide synthase/nonribosomal peptide synthetase of *Microcystis aeruginosa*, cyanobacterial hydrogenase genes in *Anabaena* sp. and *GTPase/ATPase* genes in *Synechocystis* sp (Kaebernick et al., 2002). Thus, the RT-PCR was used to detect mRNA transcripts in *ack* and *pta* gene in this study. The PCR products from RT-PCR of these genes were greater in cells grown under phosphate-limiting condition than in normal BG-11 (Fig. 43). The results showed that the acetate kinase and phosphotransacetylase synthesis occur at least at the mRNA level. Moreover, these genes are inducible by phosphate stress. The transcription regulation of *ack* and *pta* genes was also examined in *Methanosarcina thermophila*. The levels of transcripts from Northern blot analysis were several fold greater in acetate grown cells than in cells grown on other substrates such as mehanol, trimethylamine, dimethylamine and monomethylamine (Singh-Wissmann and Ferry, 1995). These results showed that the *pta* and *ack* gene expression is regulated by the growth condition not only the carbon source but also

phosphate concentration in the media.

In *Methanosarcina thermophilla*, only one copy of each gene is present per chromosome as confirmed by Southern analysis (Singh-Wissmann and Ferry, 1995). The data from a Cyanobase search showed one copy of *ack* and *pta* genes in *Synechosytis* sp. PCC 6803. As a simple method to confirm that there is only one copy of each gene, the *ack* and *pta* interruption strains were measured for the acetate kinase and phosphotransacetylase activities, respectively. If there is only one copy of these genes per genome in *Synechocystis* sp. PCC 6803, the activities of Δ*sll1299* and Δ*slr2132* should not be induced under both normal BG-11 and phosphate-limiting BG-11.

There are some reports about the organization of *ack* and *pta* gene in *Methanosarcina thermiphila* and *Sinorhizobium melioti* (Singh-Wissmann and Ferry, 1995 and Summer et al., 1999). The *pta* gene is located upstream and djacent to *ack* gene in both organisms. In addition, the *pta* interruption strain in *Sinorhizobium meliloti* eliminates the ack activity because these two genes are transcribed as an operon from a promoter located upstream of *pta*. On the other hand the organization of these genes in the *Synechocystis* sp. PCC 6803 genome from cyanobase showed that the sll1299 is not adjacent to slr2132 gene. If the acetate kinase activity was not induced in the phosphate-limiting condition in the *pta* interruption strains in *Synechocystis* sp. PCC 6803. It may be explain that the expression of *ack* and *pta* genes are not transcribed as an operon from promoter located upstream of *pta*.

In all eubacteria, acetyl-CoA is converted to acetate by the classical mechanism involving two enzymes, phosphotransacetylase and acetate kinase (Bock et al., 1999).

Acetate also serves as substrate of catabolism and anabolism in several aerobic and anaerobic prokaryotes. The activation of acetate to acetyl-CoA, is the first step prior to its utilization in metabolism (Bock et al., 1999). To date acetate kinases have been purified from various bacteria and from the archeon *Methanosarcina thermophilla*. However, this enzyme has not yet been isolated and characterized from cyanobacteria. The partial purification and characterization of acetate kinase from *Synechocystis* sp. PCC 6803 was performed in this study. Partially purified acetate kinase was obtained after 30% ammonium sulfate and phenyl-sepharose. The phenyl-sepharose is the hydrophobic interaction chromatography (HIC). The separation bases on reversible hydrophobic interaction between immobilized hydrophobic ligands on chromatographic medium and non-polar regions on the surface of proteins. Adsorption of proteins to a HIC adsorbent is favored by a high salt concentration in the mobile phase because the ionic strength of solution controls the protein hydrophobicity and therefore its degree of adsorption. The elution of acetate kinase is accomplished by decreasing the ammonium sulfate concentration (Leksakorn, 2001). When the data of purification were analyzed, it was found that the enzyme was purified to 6.18 folds and the specific activity was 2.48 unit/mg protein with 64.8 %yield. The molecular weight of acetate kinase from *Syenechocystis* sp. PCC 6803 is about 48 kDa corresponding to one of the protein bands from the last purification step (Fig. 45).

An activity stain specific for acetate kinase after separation by electrophorysis in native polyacrylamide gels was performed to examine the native enzyme. The test system was based on the reduction by NADPH+H^+ of nitro blue tetrazolium via phenanzine methosulfate. Enzymic activity after electrophoresis was detected with enzyme from various organisms including *E. coli* and *Acetobacterium woodii* (crude

extracts and purified enzyme) (Winzer et al., 1997). There is only single purple band from the activity stain in each purification step (Fig. 46). This suggested that partially purified acetate kinase represented the native acetate kinase. Most of acetate kinases from eubacteria and the archeon *M. thermophilla* are typically a homodimeric structure. Exceptions are the acetate kinase of *Clostidium thermoaceticum, Bacillus stearothermophillus* (Nakajima et al., 1978) and *Desulfovibrio vulgaris* (Yu et al., 2001), which have been reported to be monomeric, homotetrameric and heterodimeric enzymes, respectively (Bock et al., 1999). Therefore gel filtration chromatography may further determine subunit size of acetate kinase from *Synchocystis* sp. PCC 6803.

The optimum temperature of acetate kinase catalysis is 40 °C (Fig. 47) whereas this enzyme in *Thermotoga maritima* (Aceti and Ferry, 1988) showed an optimum at 90°C. The acetate kinase showed similar activity between pH 6.5-8.5 (Fig. 48). The results suggested that this enzyme catalyses reactions over a broad pH range. Acetate kinase of *Desulfovibrio vulgaris* (Yu et al., 2001) also showed high activity over a broad pH range of 7.0-8.5. These data are different from those seen in *Thermotoga maritima* (Bock, et al., 1999), and *Methanosarcina themophilla* (Aceti and Ferry, 1988).

Like all known acetate kinases, *Synechocystis* etc enzyme requires divalent cations for activity such as Mg^{2+} providing maximum activity in most cases. This enzyme has an optimal Mg^{2+}/ATP ratio of 1:1 (Fig. 49), suggesting that Mg^{2+} is required only to complex ATP rather than to have additional effects on enzyme function or stability. A 1:1 ratio has also been determined for the enzymes of *Escherichia coli* (Anthony and Leonard, 1971), *Salmonella typhimurium* (Fox and

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Roseman, 1986) and *M. thermophila* (Aceti and Ferry, 1988). For acetate kinase from *C. acetobutylicum*, an optimal Mg^{2+}/ATP ratio of 2 has been reported (Diez-Gonzalez et al., 1997). Furthermore, the substrate specificity was determined in the direction of acetyl phosphate formation. The enzyme was highly active with acetate, whereas the reaction rates with propionate and butyrate were low. The acetate kinase also prefered ATP as the phosphate donor for acetate phosphorylation (Table 8).

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