CHAPTER 6
DISCUSSION

Fossils of cyanobacteria are among the oldest on record, some dating back 3.5 billion years. The discovery of a dual-specificity protein phosphatase, IphP, possessing the HAT motif and encoded within the genome of a cyanobacterium raised new questions as to the origin and role of protein tyrosine phosphorylation in nature (1). The goal of the research described herein was to extend our knowledge of protein tyrosine phosphorylation in these ancient organisms. To this end, the substrate specificity and catalytic properties of IphP were examined in detail. Also, the presence of tyrosine phosphorylated proteins, as well as the activity of a protein tyrosine kinase, was demonstrated in *Anabaena* PCC 7120. Finally, a dual-specificity protein phosphatase was partially purified from this cyanobacterium and characterized.

Specificity and Catalytic Properties of IphP

A phosphatase whose *raison d'etre* is to scavenge phosphate for nutritional purposes would be expected to readily hydrolyze, showing little selectivity or preference, a diverse assortment of low molecular weight- and protein substrates. A regulatory PTP, on the other hand, would most likely display a preference for phosphoproteins over low molecular weight organophosphates and demonstrate selectivity among potential substrates . The catalytic properties of IphP were therefore examined in detail in an attempt to surmise the probable role of the phosphatase *in vivo*.

IphP dephosphorylated a number of proteins and peptides which had been phosphorylated on serine, threonine, or tyrosine residues, including RCM-lysozyme, MAPK, casein, poly (Glu,Tyr), myelin basic protein, YINAS, and angiotensin II. In contrast, the enzyme displayed no detectable activity toward histone H2a, histone H2b, polyphosphohistidine, polyphospholysine, or kemptide. Kinetics experiments revealed a preference on the part of IphP for protein and peptide substrates over low molecular weight organophosphate substrates. The K_M values for protein and peptide substrates were ~10-fold lower than those obtained for low molecular weight organophosphate substrates. Likewise, the k_{cat}/K_M values for protein and peptide substrates were generally higher than those observed for low molecular weight organophosphates, especially when limiting the samples considered to those compounds most likely to encountered by IphP in nature. The greater efficacy of IphP toward macromolecular substrates over low molecular weight compounds and the fastidious nature of IphP refute the argument that the enyzme hydrolyzes bound phosphate for nutritional purposes, suggesting instead a regulatory role for the enzyme, i.e.,

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[1]Potts, M., H. Sun, K. Mockaitis, P. J. Kennelly, D. Reed, and N. K. Tonks. (1993). A protein-tyrosin / serine phosphatase encoded by the genome of the *cyanobacterium Nostoc* commune UTEX 584. *J. Biol. Chem*. **268**: 7632-35.

that targeted protein substrates probably exist *in vivo*.

Heparin was found to effect IphP activity in a substrate-dependent manner. The polyanion stimulated IphP activity nearly 10-fold toward casein (P-Ser) and MAPK (P-Thr/P-Tyr), but inhibited its activity toward the other protein substrates examined. This phenomenon was attributed to the ability of heparin to recruit IphP and these substrates to the same microenvironment. Complexes between heparin and casein (P-Ser), MAPK (P-Thr/P-Tyr), or IphP were detected by sucrose density gradient ultracentrifugation and / or heparin-agarose affinity chromatograpy. Current models of signal transduction predict that many protein phosphatases are "regulated" by virtue of their location within the cell, rather than by modulation of enzyme activity (2). These data suggest that IphP may be regulated in such a manner, being concentrated about a macromolecular polyanion or attracted to proteins bearing a net negative charge.

Detection of Phosphotyrosyl-phosphoproteins in *Anabaena* PCC 7120

The discovery of IphP, a genomically-encoded dual-specificity protein phosphatase, in *Nostoc commune* UTEX 584 inspired the search for components of a tyrosine phosphorylation network in *Anabaena* PCC 7120, a cyanobacterium amenable to genetic manipulation. Such a network would minimally consist of at least one tyrosine-phosphorylated protein, a protein-tyrosine kinase activity, and a protein-tyrosine phosphatase activity.

Cross reactions between anti-phosphotyrosine antibodies and *Anabaena* proteins were observed in initial investigations in Malcolm Pott's laboratory, strongly suggesting the presence of tyrosine-phosphorylated proteins in this organism. Here, we confirmed this conclusion using radioisotopic and enzymatic methods.

Proteins in concentrated crude extracts of *Anabaena* PCC 7120 were radiolabelled *in vitro* by incubation with [^{32}P]-ATP. Two forms of the compound, [α-^{32}P]-ATP and [γ-^{32}P]-ATP, were utilized to determine the nature of the protein-bound radiolabel: adenylated proteins would be labelled by both forms of ATP, whereas phosphorylated proteins would only be labelled by the [γ-^{32}P]-ATP. Numerous proteins were observed to be labelled upon incubation with [γ-^{32}P]-ATP; however, no proteins appeared to be labelled upon incubation with [α-^{32}P]-ATP. Thus, it was concluded that the radiolabelled proteins were phosphorylated.

Protein-bound [^{32}P]phosphate was liberated from several of the labelled proteins by the tyrosine-specific protein phosphatase PTP1B, indicating that at least a portion of the radioloabel incorporated into those proteins was in the form of phosphotyrosine. In addition, phosphoamino acid analyses revealed the presence of phosphotyrosine on proteins of 27kDa and 21kDa. These results substantiate the western blot data and confirm the presence of

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[2]Tonks, N. K. (1990). Protein phosphatases: key players in the regulation of cell function. *Curr. Opinion in Cell Biol*. **2**: 1114-24.

tyrosine-phosphorylated proteins in *Anabaena* PCC 7120. Furthermore, the presence of a tyrosine kinase activity(ies) was made manifest by the detection of these tyrosine-phosphorylated proteins, since no exogenous kinases were added to the extracts.

Characterization of a DSP from *Anabaena* PCC 7120

The presence of a tyrosine kinase activity and tyrosine-phosphorylated proteins in *Anabaena* PCC 7120 implied the presence of a protein tyrosine phosphatase activity in this organism. When soluble proteins from cell extracts were examined for a PTP activity toward the artificial substrate RCML (^{32}P-Tyr), an activity was apparent with optimal catalysis occuring at pH 7.5. The apparent PTP activity was partially purified by chromatographic means. Several lines of evidence verified that the partially purified enzyme was a phosphatase rather than a protease:

1. the partially purified enzyme hydrolyzed low molecular organophosphates, such as pNPP;
2. the radioactive product derived from [^{32}P]-labelled phosphoproteins was soluble in a solution of 4% (w/v) charcoal; and
3. the radioactive product was determined to be $^{32}PO_4$ by a molybdic acid extraction procedure.

The physical and catalytic properties of the partially purified protein phosphatase were examined. The soluble enzyme was determined to exist as a monomer of ~38kDa by SDS-PAGE and sucrose gradient density ultracentrifugation. The phosphatase possessed an isoelectric point of ~6.5, was most active in the neutral pH range, and displayed pNPPase activity at temperatures as high as of 70^{o}C. The enzyme exhibited dual-specificity *in vitro*, dephosphorylating RCML (P-Ser), RCML (P-Tyr), and casein (P-Ser). However, the enzyme displayed no detectable activity toward phosphorylase a (P-Ser), thus demonstrating a potential for selectivity among potential protein substrates.

The partially purified protein phosphatase shared the same inhibition profile as IphP and other well-characterized dual-specificity protein phosphatases. PTP activity was inhibited by the sulfhydryl-modifying reagents NEM, IAA, HMPSA, and HMB and the PTP inhibitors sodium orthovanadate and heparin. In contrast, the enzyme was unaffected by EDTA, EGTA, tetramisole, sodium potassium tartrate, okadaic acid, or microcystin-LR, inhibitors of acid phosphatases, alkaline phosphatases, and eukaryotic Ser/Thr protein phosphatases.

While protein kinases usually display restricted specificity toward one or a few protein substrates, protein phosphatases often act upon many phosphoproteins (3). Consequently, the activity of a protein phosphatase in nature is sometimes limited by

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[3]Stone, R. L. and J. E. Dixon. (1994). Protein-tyrosine phosphatases. *J. Biol. Chem*. **269**: 31323-26.

restricting the enzyme to a certain tissue type and / or a specific subcellular location or compartment (4). Since the *Anabaena* DSP was partially purified from cells grown in BG11 media, the enzyme is apparently present in the vegetative cells of *Anabaena* PCC 7120. It is currently not known if the DSP is restricted to the these cells or whether the protein phosphatase is also present in other cell types (e.g., heterocysts). When vegetative cells were separated into membrane, cytoplasmic, and periplasmic fractions, the enzyme was detected exclusively in the periplasmic fraction. The enzyme was thus assigned the title PAD, for Periplasmic *Anabaena* DSP.

The activities of regulatory enzymes are themselves often modulated by allosteric effectors or environmental parameters. Several small metabolites and environmental factors were surveyed as effectors of PAD activity. None of the metabolites examined *in vitro* had an effect on PAD activity. However, PAD activity was discovered to vary about 5-fold over time in a rhythmic fashion *in vivo*. Peaks of activity were observed following transitions from light-to-dark and dark-to-light when cells were entrained to a 12 hour light cycle. The rhythmic fluctuations of PAD activity appeared to be independent of the light signal, however, since they persisted for at least 96 hours under continuous light. This is not unlike the rhythmic nitrogenase activity found in other cyanobacteria, which is known to be active only in the dark when cells are entrained to a light / dark cycle, but remains rhythmic nonetheless when cells are released into continuous light (5).

Given the subcellular location of PAD, a search was launched for periplasmic phosphoproteins which might serve as physiological substrates for this protein phosphatase. Periplasmic proteins were labelled *in vitro* by incubation in the presence of [γ-^{32}P]-ATP. Several radiolabelled proteins were resolved by SDS-PAGE. The radiolabel incorporated into a protein of ~55kDa, determined by phosphoamino acid analysis to be modified on serine, was reduced upon incubation of labelled periplasmic proteins with PAD. A periplasmic protein of ~55kDa was also radiolabelled *in vivo* upon incubation of cells with $^{32}PO_4$, and presumably is the same protein that was labelled *in vitro*. The amount of radiolabel associated with this protein, and others, varied inversely with PAD activity over a 12 hour period. Future experiments might include purification and amino acid sequencing of this phosphoprotein, as this could provide insight into the role of PAD and the nature of the signal being transduced.

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[4]Walton, K. M. and J. E. Dixon. (1993). Protein tyrosine phosphatases. *Annu. Rev. Biochem*. **62**: 101-20.

[5]Schneegurt, M. A., D. M. Sherman, S. Nayar, and L. A. Sherman. (1994). Oscillating behavior of carbohydrate granule formation and dinitrogen fixation in the cyanobacterium *Cyanothece* sp. strain ATCC 51142. *J. Bacteriol*. **176**: 1586-97.

Conclusions

The recent discoveries of protein-tyrosine kinases, tyrosine phosphorylated proteins, and protein-tyrosine phosphatases in numerous bacteria imply an ancient history for tyrosine phosphorylation as a regulatory mechanism, despite the previously held assumption that this mode of regulation developed late in biological history. The detection of such components in cyanobacteria further emphasizes the antiquity of tyrosine phosphorylation, since these organisms are believed to be among the earliest inhabitants of Earth. Purification and genetic manipulation of these components should provide much insight into the details and physiological role(s) of this regulatory mechanism in *Anabaena* PCC 7120. In addition to revealing how tyrosine phosphorylation is exploited in this biochemically and morphologically complex cyanobacterium, such studies may provide clues concerning the origins and developement of this mode of regulation throughout nature.