CHAPTER 5
PARTIAL PURIFICATION AND CHARACTERIZATION OF A SOLUBLE 38kDa DSP

Objectives:

1. Determine if *Anabaena* PCC 7120 contains an enzyme activity capable of liberating ^{32}P radioactivity from radiolabelled phosphoproteins;
2. Determine whether the release of ^{32}P from radiolabelled phosphoproteins is due to a dephosphorylation event;
3. Partially purify the prominent PTP activity for *in vitro* characterization;
4. Identify possible physiological substrates for the prominent PTP.

Rationale:

Tyrosine-phosphorylated proteins were detected in extracts of *Anabaena* PCC 7120. The existence of these phosphoproteins implies the presence of protein tyrosine kinase(s) and protein tyrosine phosphatase(s) in this organism. In this study, we attempted to isolate and characterize a PTP activity in *Anabaena* PCC 7120.

Detection of PTP Activities in Extracts of *Anabaena* PCC 7120

Concentrated, cell-free extracts of *Anabaena* PCC 7120 were tested for PTP activity toward the artificial substrate RCML (^{32}P-Tyr). Protein phosphatase assays were performed at pH 5.0 (the optimum pH of IphP activity) and at pH 7.0 (Fig. 5.1A). Significantly more PTP activity was observed at pH 7 than at pH 5. Following ultracentrifugation, an apparent protein phosphatase activity could be detected in both the soluble and membrane fractions (Fig. 5.1B). The putative PTP activity in the soluble fraction was partially purified for subsequent characterization.

Partial Purification of a Soluble PTP

Cells from semi-continuous cultures, typically 10-15g wet weight, were harvested as described under Materials and Methods, resuspended in 3-4 volumes of lysis buffer and lysed by passage through a French pressure cell as described under Materials and Methods. The cell-free extract was subjected to 1 hour of ultracentrifugation at 100,000 x *g* at 4°C. The supernatant liquid (40-50 mL containing 350-500mg of protein) was collected as the soluble fraction.

Several chromatographic resins, including heparin-agarose, carboxy methyl cellulose, Sephacryl S-200, and phenyl sepharose, were tested for purification of the soluble PTP activity, but rejected because of low recovery. However, a significant enrichment of PTP activity was achieved when the soluble fraction was passed through a column of DE52

cellulose. Twenty-five milliliters of the soluble fraction was applied to a column of DE52 cellulose (5 x 20 cm) that had been equilibrated in 50mM Tris, pH 7.5, 50mM NaCl, 1mM DTT (DE52 equilibration buffer). The column was washed with an additional 1000mL of DE52 equilibration buffer. Protein peaks were pooled and examined for PTP activity toward RCML (^{32}P-Tyr) (Fig 5.2). Pooled fractions containing PTP activity were concentrated to 3-5mL and sterilized by passage through a 0.2 micron filter. This fraction, which exhibited a distinguishable brown color, was designated the DE52 fraction. The column was regenerated by washing with 500mL of 50mM Tris, pH 7.5, 1mM DTT, 0.5M NaCl, then 500mL of DE52 equilibration buffer. The purification process was repeated for the remaining 25mL of the soluble fraction and the two DE52 fractions were combined into a single fraction. The entire DE52 fraction (~10mL) then was loaded onto a cellulose phosphate column (1.5 x 12 cm) equilibrated with 50mM Tris, pH 7.5, containing 50mM NaCl and 1mM DTT (CP equilibration buffer). Cellulose phosphate functions as both a cation exchange resin and as an affinity resin for phosphate-binding proteins. The column was washed with 30mL (~1 column volume) of CP equilibration buffer, then 30mL of 50mM Tris, pH 7.5, 1mM DTT, 100mM NaCl, and finally one column volume of 50mM Tris, pH 7.5, 1mM DTT, 300mM NaCl (Fig 5.3). As expected, the PTP was not eluted until the column was developed with a buffer containing high salt concentrations (i.e, 300mM NaCl). Fractions containing PTP activity were pooled, concentrated to a volume of 3-5mL, and sterilized by passage through a 0.2 micron filter. This fraction was designated the CP fraction. Following cellulose phosphate chromatography, it was estimated that the PTP had been enriched over 1000-fold with about 20% recovery of activity and 0.02% recovery of total protein (Table 5.1). Each week, as cells were freshly harvested, the purification procedure was repeated and the final fractions pooled.

The partially-purified enzyme could be kept at room temperature overnight or at 4^{o}C for several months with no detectable loss of activity. The sterilized DE52 and CP fractions were routinely stored at 4^{o}C.

Is the Enzyme in the DE52 Fraction a PTP?

RCML (^{32}P-Tyr) was utilized as the substrate for protein phosphatase assays throughout the purification procedure of the soluble PTP. Protein phosphatase assays employing radiolabelled protein substrates are commonly terminated by precipitation of substrate proteins with cold TCA. However, TCA-soluble ^{32}P can be produced by either dephosphorylation of the labelled protein to yield inorganic phosphate or by proteolysis of the labelled protein into small, acid soluble peptides. The following precautions were taken, therefore, to ensure that the release of ^{32}P was due specifically to dephosphorylation of the tyrosine residue of RCML (^{32}P-Tyr): 1, the assays were always terminated using a suspension of Norit A charcoal, which absorbs both proteins and peptides, and 2,

phosphatase assays were usually performed in the presence of EDTA and PMSF, potent inhibitors of proteases, and EGTA which is known to inhibit a Ca^{++}-dependent *Anabaena* protease. In addition, the partially- purified PTP efficiently hydrolyzed pNPP and other low molecular weight organophosphates (Table 5.2). More importantly, the reaction product was demonstrated to be $^{32}PO_4$ by extraction into organic solvents as a molybdic acid complex (Fig. 5.4).

In Vitro Characterization of the Soluble PTP

During the course of purifying the soluble PTP activity, a novel "in-gel" protein phosphatase assay was described by Gates and coworkers (1). I employed this technique to examine the soluble, DE52, and CP fractions (Fig. 5.5). Only one PTP activity was evident in each fraction. In each instance this activity migrated with an apparent molecular mass of ~38kDa.

Because the in-gel assay involves a denaturation step (i.e., SDS-PAGE), the native molecular weight of the enzyme was determined by sucrose gradient density ultracentrifugation. The molecular weight of the PTP was judged to be 33kDa by this procedure (Fig. 5.6), indicating that the native enzyme exists as a monomer. In addition, only one peak of phosphatase (i.e., pNPPase) activity was detected, suggesting that the fraction was free of other phosphatase activities.

The optimum pH for catalysis was determined for both a protein substrate, RCML (^{32}P-Tyr), and a low molecular weight organophosphate, pNPP (Fig. 5.7). The enzyme was active toward pNPP over a broad pH range, a common characteristic of PTPs. In contrast, the PTP displayed activity toward RCML (^{32}P-Tyr) over a much narrower pH range, with optimal activity occuring at ~pH 7.5. With both substrates, activity declined dramatically above pH 8.0.

Many protein phosphatases require divalent metal ions for optimal activity. The effect of monovalent, divalent, and trivalent metal ions on the activity of the soluble protein phosphatase was examined (Table 5.3). All of the metals tested, with the exception of Mg^{2+}, caused some inhibition of the enzyme's activity toward RCML (^{32}P-Tyr). The metal ions Ni^{2+}, Zn^{2+}, Cu^{3+}, and Fe^{3+} elicited the most dramatic inhibitory responses. $MgCl_2$ had no effect on the PTP activity.

The isoelectric point of the soluble PTP was determined by use of the in-gel assay following 2D-PAGE (Fig. 5.8). The pI was estimated to be ~6.5.

The phosphoamino acid specificity of the soluble protein phosphatase was tested

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[1]Gates, R. E., J. L. Miller, and L. E. King, Jr. (1996). Activity and molecular weight of protein tyrosine phosphatases in cell lysates determined by renaturation after gel electrophoresis. *Anal. Biochem.* **237**: 208-15.

using a single protein, RCML, phosphorylated on either serine residues or tyrosine residues. As shown in Figure 5.9A, both substrates were dephosphorylated in the presence of the DE52 fraction in a time-dependent manner. In order to determine whether both substrates were being dephosphorylated by the same enzyme, the activity toward each was examined using the in-gel assay (Fig. 5.9B). A single band of clearing was observed in both gels at ~38kDa, indicating that the 38kDa protein phosphatase was responsible for dephosphorylation of both substrates. The 38kDa soluble protein phosphatase can thus be classified as a dual-specificity protein phosphatase (DSP).

Eukaryotic protein tyrosine phosphatases and dual-specificity protein phosphatases share a common motif (the HAT motif) possessing a catalytically relevant cysteine residue. As a result, they are sensitive to sulfhydryl-modifying reagents. The effect of several cysteine-modifying chemicals, as well as the effect of well-characterized protein phosphatase inhibitors, on the activity of the *Anabaena* DSP was examined (Fig. 5.10). The activity of the DSP toward RCML (^{32}P-Tyr) was noticeably inhibited by the sulfhydryl-modifying reagents N-ethylmaleimide (NEM), iodacetic acid (IAA), *p*-hydroxymercuriphenyl sulfuric acid (HMPSA), and *p*-hydroxymercuribenzoate (HMB). Enzyme activity was unaffected by the metal chelators, EDTA or EGTA, which inhibit the Mg^{2+}-dependent protein phosphatase PP2C, the Ca^{2+}-dependent protein phosphatase 2B, or the Mn^{2+}-dependent prokaryotic PP1s. Neither was it inhibited by tetramisole, a more specific inhibitor of alkaline phosphatases, or sodium potassium tartrate, an inhibitor of acid phosphatases. Enzyme activity was also insensitive okadaic acid or mycrocystin-LR, potent inhibitors of eukaryotic PP1 and PP2A. In contrast, enzyme activity was significantly inhibited by sodium orthovanadate and heparin, inhibitors of most well-characterized PTPs and DSPs.

Subcellular Localization of the DSP

During the course of these investigations it was noted that the freeze-thawing cell pellets of *Anabaena* PCC 7120 released proteins into the buffer solution. When freeze-thawed cells were pelleted by low-speed centrifugation, the supernatant liquid was blue. Since whole-cell lysates obtained by passage through a French pressure cell were deep red in color, it was suspected that freeze-thawing disrupted the cell wall structure, leading to the preferential release of periplasmic proteins. The fact that a significant portion of the soluble DSP activity was recovered in this blue supernatant led to the hypothesis that the 38kDa DSP might be localized to the periplasm. To test this hypothesis, an alternative fractionation procedure was employed in an attempt to disrupt the cell wall without lysing the cell membrane.

Cellular fractionation was achieved by incubation with lysozyme in a hypertonic

buffer solution (2). Freshly-grown cells were harvested by centrifugation and immediately resuspended in 30mL of lysis buffer (50mM Tris, pH 7.5, 1mM EDTA, 1mM EGTA, 1mM DTT, 0.5mM PMSF) containing 15% (w/v) sucrose. DNase II (~50µg) and lysozyme (5mg) were added and the suspension was incubated for 2 hours at 30ºC. As a control, half (15mL) of the mixture was passed through a French pressure cell to completely lyse the cells. Insoluble debris was removed from the lysate by low-speed centrifugation (2500 x *g* for 15 minutes at 4ºC) and the supernatant was collected as the cell lysate fraction. The lysozyme-treated sample was subjected to centrifugation at 2500 x g for 15 minutes at 4ºC to remove the insoluble debris and intact spheroplasts and the supernatant was collected as the periplasmic fraction. The spheroplasts were resuspended in 15mL of lysis buffer containing 15% (w/v) sucrose and lysed by 3 passes through a French pressure cell (~10k psi). Insoluble debris was removed by low-speed centrifugation (2500 x g for 15 minutes at 4ºC) and the supernatant was collected as the spheroplast lysate. Each fraction was then assayed for PTP activity using both soluble assays and the in-gel technique. To appraise the efficacy of the fractionation procedure, each fraction was assayed for the cytoplasmic marker enzyme, isocitrate dehydrogenase (IDH).

IDH activity was detected in both the cell lysate and spheroplast lysate fractions (Fig. 5.11). In contrast, no detectable IDH activity was found in the periplasmic fraction, indicating that the periplasmic fraction contained little or no cytoplasmic material.

PTP assays indicated that the majority of the protein tyrosine phosphatase activity was recovered in the periplasmic fraction, with only minor levels of activity present in the spheroplast lysate fraction (Fig.5.12). When each fraction was examined using the in-gel assay, it was apparent that the 38kDa protein phosphatase was located in the periplasmic fraction (Fig. 5.13). Based on these results, the protein phosphatase was given the name PAD (Periplasmic *Anabaena* DSP).

In Vivo PAD Activity is Rhythmic

Nitrogen fixation and / or photosynthesis must be carefully controlled in order for the cell to maintain a constant C/N ratio. Many low molecular weight metabolites serve as indicators of carbon and nitrogen availibility or flux through metabolic pathways. The ratio of glutamate to α-ketogluterate, for example, is monitored by the cell to ascertain the

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[2]Snyder, W. B. and T. J. Silhavy. (1995). β-galactosidase is inactivated by intermolecular disulfide bonds and is toxic when secreted to the periplasm of *Escherichia coli. J. Bacteriol*. **177**: 953-63.

availibility of combined nitrogen (3). The ratio is usually maintained at about 1:1. Should the ratio increase, the cell must respond by initiating nitrogen fixation and / or slowing growth and photosynthesis. The mechanism(s) behind sensing and responding to changes in metabolite concentration is largely unknown.

Several low molecular weight compounds of physiological relevence to cyanobacteria were examined as effectors of PAD activity *in vitro*. As shown in Table 5.4, none of the compounds examined exhibited a measurable effect on the enzyme. This was especially surprising for the phosphosugars, as they were expected to act as competitive inhibitors, if not allosteric effectors, of enzyme activity.

Light is an extremely important environmental parameter to cyanobacteria. *Anabaena* PCC 7120, for example, is an obligate photoautroph and must obtain light energy to survive. In the laboratory, as in nature, cyanobacteria are typically exposed to light / dark cycles of approximately equal duration. Since photosynthesis cannot proceed in the dark, the cells must sense and respond to changing light conditions, as to changing nutrient conditions, in order to maintain a proper C/N ratio.

The effect of light on PAD activity was examined by harvesting the periplasm from a portion of a cell culture exposed to a 12hr:12hr light/dark cycle at incremental time points and measuring the PTP activity at pH 7.5. During the course of one light /dark cycle, PAD activity varied ~5-fold and demonstrated peaks of activity immediately following a light-to-dark or dark-to-light transition (Fig. 5.14). However, the fluctuation in PAD activity was not directly dependent on a light signal, since rhythmic activity persisted for up to 96 hours in continuous light (Fig. 5.15).

Dephosphorylation of a Periplasmic Phosphoprotein by PAD *in vitro*

Like other dual-specificity protein phosphatases, PAD dephosphorylates both protein- and low molecular weight substrates. Given its periplasmic location, the phosphatase could potentially function as either a regulatory protein phosphatase or a phosphate scavenger. It was asked, therefore, if PAD could dephosphorylate cyanobacterial phosphoproteins, as would be expected if they constituted natural protein substrates for the enzyme.

Potential phosphoprotein substrates were radiolabelled *in vitro* by incubation of the soluble fraction with [γ-^{32}P]-ATP (Fig. 5.16A). The labelled proteins were then treated with partially purified PAD and examined by SDS-PAGE and autoradiography for removal of incorporated radiolabel. The extent of ^{32}P-incorporated into two of the phosphoproteins, pp55 and pp30, was significantly reduced following incubation with PAD-enriched DE52

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[3]Forchhammer, K. and N. Tandeau de Marsac. (1995). Phosphorylation of the P_{II} protein (*glnB* gene product) in the cyanobacterium *Synechococcus* sp. strain PCC 7942: analysis of in vitro kinase activity. *J. Bacteriol*. **177**: 5812-17.

fraction (Fig. 5.16A). These data suggested that pp55 and / or pp30 may be physiological substrates for PAD. (It should be noted that the radiolabel associated with several proteins was not diminished upon incubation with DE52 fraction. Assuming that these proteins were modified by phosphorylation (see Fig. 4.1), this result further indicates a selective behavior by PAD.)

In order to act as substrates *in vivo*, phosphoproteins must be accessible to the protein phosphatase. Since PAD was localized to the periplasm, it was asked if pp30, pp55, or both were also located in the periplasmic fraction. To determine their locations, the periplasmic fraction was isolated prior to labelling the phosphoproteins with ^{32}P. As shown in lane 1 of Figure 5.16B, a protein of ~55kDa was again radiolabelled, indicating that this protein, and the protein kinase that phosphorylated it, were located in the periplasm. In contrast, the 30kDa phosphoprotein visible in the soluble fraction was no longer present, suggesting that the protein is either cytoplasmic or could not be phosphorylated in the absence of cytoplasmic material. Upon incubation of the labelled periplasmic proteins with the DE52 fraction, the amount of ^{32}P-incorporated into pp55 was again found to decrease (Fig. 5.16B, lane 2). In addition, a high molecular weight periplasmic protein (~120kDa) was dephosphorylated upon incubation with PAD. Based on these results, the 55kDa and 120kDa phosphoproteins appear to be the best candidates as physiological substrates for PAD.

Phosphoproteins were labelled *in vivo* by incubating cells of *Anabaena* PCC 7120 with [^{32}P]orthophosphate. Not unexpectedly, the number and size of phosphoproteins observed in the periplasmic fraction differed somewhat from those observed by labelling *in vitro* with [^{32}P]ATP. The level of ^{32}P incorporated into periplasmic proteins was monitored over a 12 hour period, as was PAD activity. The amount of radioactivity associated with several proteins, including a protein of ~55kDa, increased over this time period while PAD activity decreased (Fig. 5.17). These data strongly suggest that the 55kDa phosphoprotein dephosphorylated by PAD *in vitro* is likely acted upon by PAD *in vivo* as well, since the phosphorylation state of this protein varies inversely with PAD activity. In addition, the reciprocal relation between global protein-associated radiolabel and PAD suggests that PAD may act upon multiple periplasmic phosphoproteins.

Table 5.1. Shown are example values obtained from a 10g (wet weight) pellet of *Anabaena* PCC 7120. Specific activity toward RCML (^{32}P-Tyr) reported as pmol/min/mg.

Table 5.1
Purification of the soluble PTP

Fraction	Volume (mL)	protein mg/mL	Specific activity	Enrich-ment	%Recovery activity	%Recovery protein
lysate	50	~20	-	-	-	-
soluble	30	10.3	105	1	100	100
DE52	3	0.39	9200	87	31	0.4
CP	3	0.02	114000	1086	20	0.02

Table 5.2. The specific (μmol/min/mg) and relative activities of the hydrolase are shown for selected organophosphates. Assays were performed for 30 minutes at 30°C in 50μL volumes of 50mM Tris, pH 7.5, containing 3.5μg of the DE52 fraction, 1mM DTT, and 5mM substrate. The production of P_i was monitored using the Malachite Green assay as described under Materials and Methods.

Table 5.2
Low molecular weight organophosphate hydrolase activity in the DE52 fraction

Substrate	Specific Activity	Relative Activity
pNPP	98.2	100
α-naphthyl phosphate	28.4	31
β-naphthyl phosphate	77.0	83
phosphoserine	27.5	30
phosphotyrosine	77.9	84

Table 5.3. Assays of the DE52 fraction (30ng) were performed under standard conditions toward RCML (P-Tyr) in the absence or presence of each of the listed metal chlorides at a final concentration of 0.5mM. a, 100%=21.7 nmol/min/mg.

Table 5.3
The effect of monovalent, divalent, or trivalent metal chlorides on the soluble PTP activity

Metal Chloride	Relative Activity
none	100[a]
NaCl	65
KCl	74
$MgCl_2$	95
$MnCl_2$	65
$CaCl_2$	64
$NiCl_2$	10
$ZnCl_2$	7
$CuCl_3$	10
$FeCl_3$	25

Table 5.4. Listed are physiologically relevent compounds tested as allosteric affectors of PAD. PTP activity assays towared RCML (^{32}P-Tyr) were performed under standared conditions (see Materials and Methods) using ~50ng of the DE52 fraction. Activity is reported relative to that observed in the absence of exogenous metabolites.

Table 5.4
The effect of key metabolites on PAD activity *in vitro*

Metabolite	Relative Activity
ammonium chloride	95
glutamate	101
α-ketogluterate	93
glucose-6-phosphate	95
glucose-1-phosphate	90
ribulose-1,5-diphosphate	90

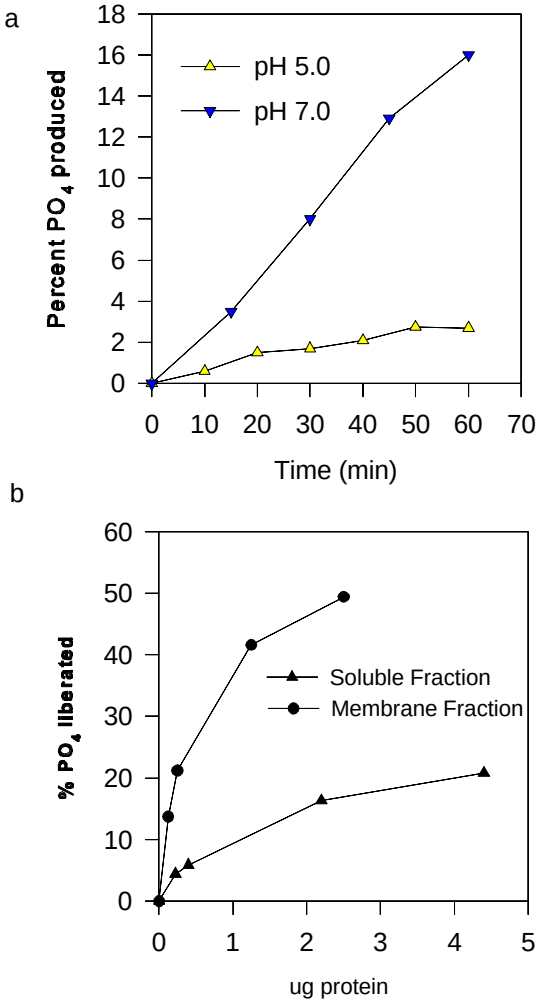

Figure 5.1

PTP activities in *Anabaena* PCC 7120. (a) Extracts, 100µg protein, were assayed in 25µL volumes of either 40mM sodium acetate, pH 5.0 or Tris, pH 7.0 containing 1mM DTT and 2µM RCML (^{32}P-Tyr) at 30° C; (b) PTP activities in the soluble and membrane fractions (obtained by ultracentrifugation at 100,000 x g for 90 minutes at 10°C). Assays were performed for 30 minutes at 30°C in 50mM Tris, pH 7.5, 1mM DTT, 2µM RCML (^{32}P-Tyr). The specific protein tyrosine phosphatase activities in the soluble and membrane fractions were 318 pmol/min/mg and 2630 pmol/min/mg, respectively.

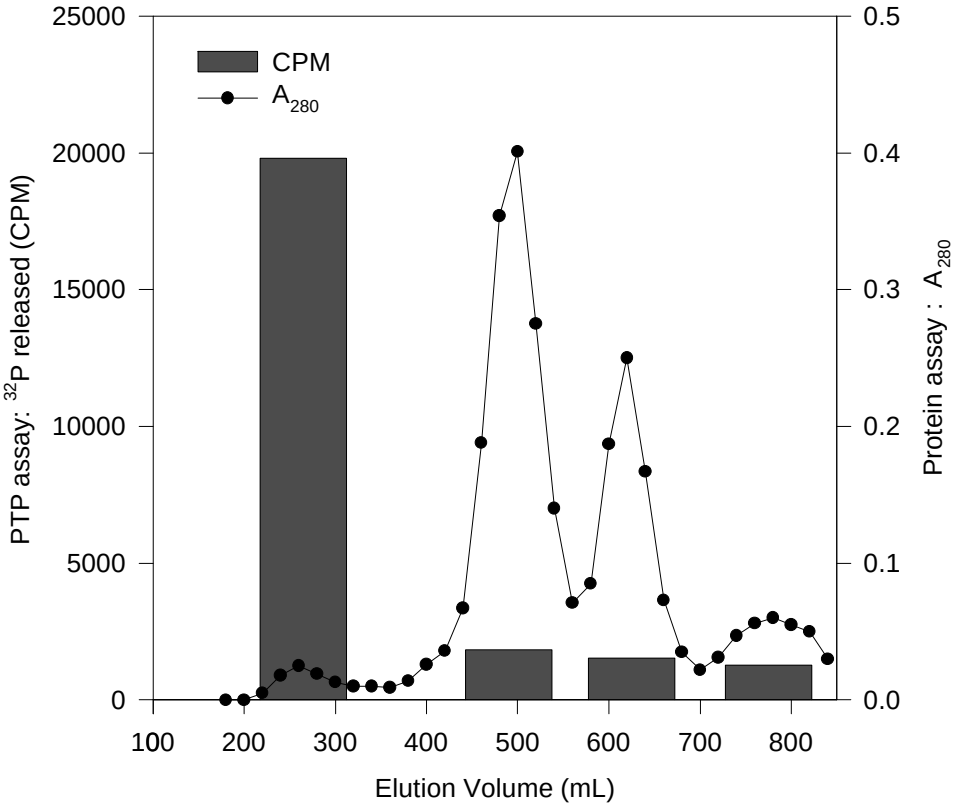

Figure 5.2

DEAE chromatography of the soluble fraction. Twenty-five mL of the soluble fraction (~300mg protein) was applied to a DEAE cellulose ion-exchange column as described in the text. Fractions, 10mL, were collected and assayed for protein using the Bradford method. Protein peaks (line graph) were pooled: peak 1, fractions 175-350; peak 2, fractions 351-550; peak 3, fractions 551-690; peak 4, fractions 691-880. The bar graph illustrates the PTP activity associated with each pooled protein peak.

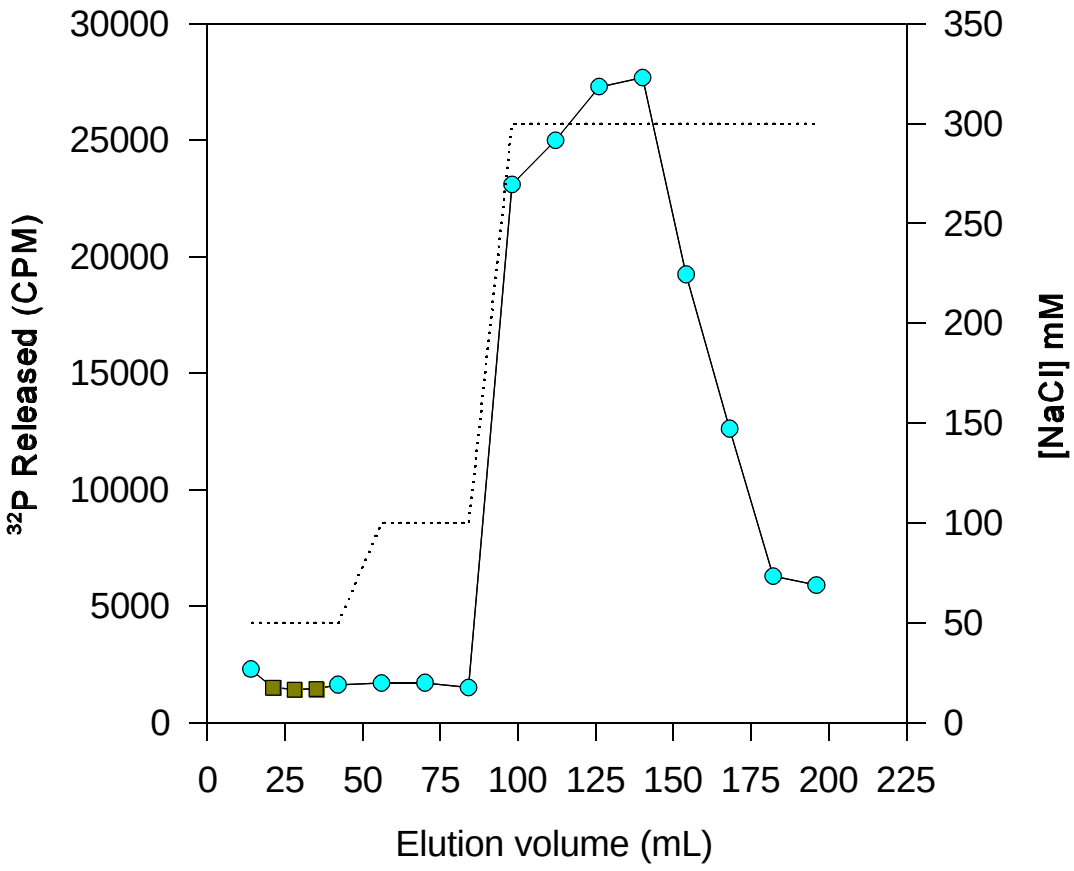

Figure 5.3

Cellulose phosphate chromatography of the DE52 fraction. Concentrated DE52 fraction (5 mL, ~1.5mg protein) was fractionated on a column of cellulose phosphate as described in the text. Shown is a graph of PTP activity vs fraction number. The concentration of NaCl is indicated by the dashed line. Fractions, 7mL, were collected and assayed for PTP activity toward RCML (^{32}P-Tyr). Active fractions were pooled, concentrated to ~5mL, and sterilized by passage through a 0.2 micron filter. Squares indicate where a brown color was discernable.

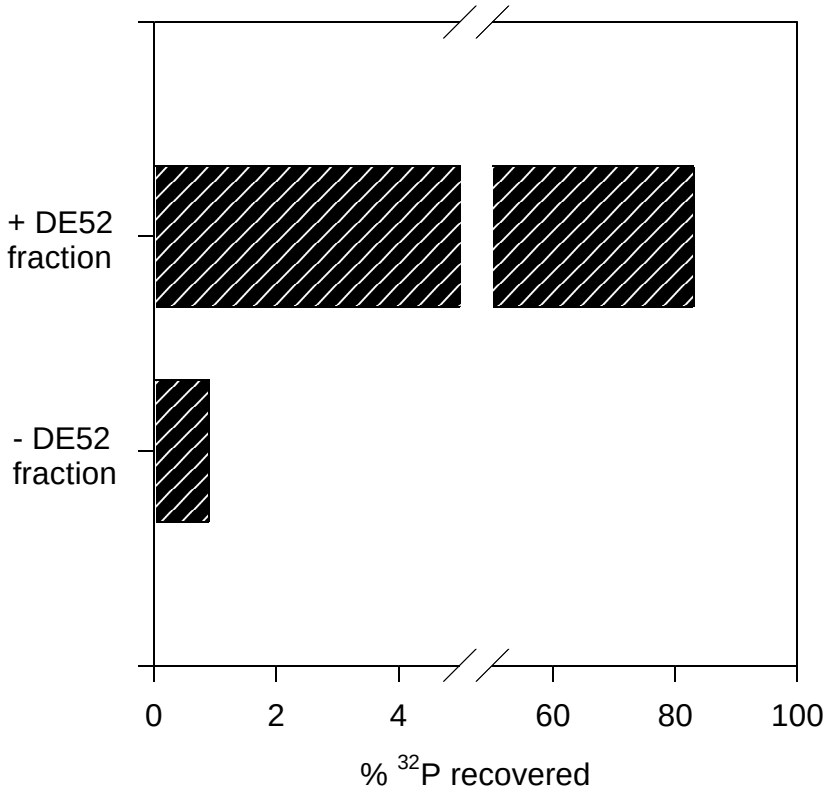

Figure 5.4

Molybdic acid extraction of inorganic [^{32}P]phosphate. The form of the radiolabelled product obtained following incubation of the DE52 fraction with RCML (^{32}P-Tyr) was assessed using the molybdic acid extraction procedure for inorganic phosphate (see Materials and Methods). Shown is the percent of radiolabel recovered by the extraction following incubation of RCML (^{32}P-Tyr), 2µM final, for 60 minutes at 30°C in 25µL of 50mM Tris, pH 7.5, 1mM DTT, 0.5mM EDTA, and 0.5mM EGTA with or without 5µg of the DE52 fraction.

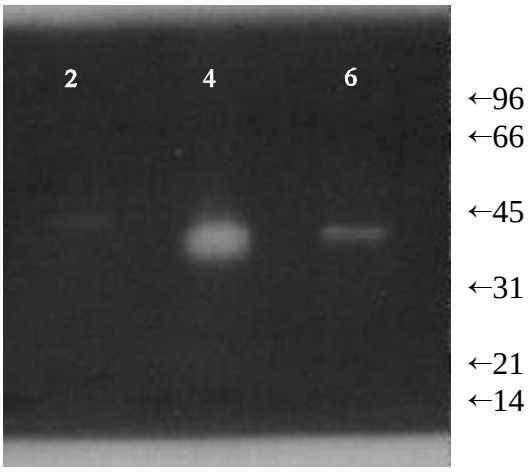

Figure 5.5

In-gel assay of soluble, DE52, and CP fractions. Samples of the active fractions from the partial purification of the soluble PTP were examined using the in-gel assay with RCML (^{32}P-Tyr) as the substrate. Lane 2 contains 10.5µg of the soluble fraction, lane 4 contains 2µg of the DE52 fraction, and lane 6 contains 0.3µg of the CP fraction. Molecular weight markers are shown on the right in kilodaltons.

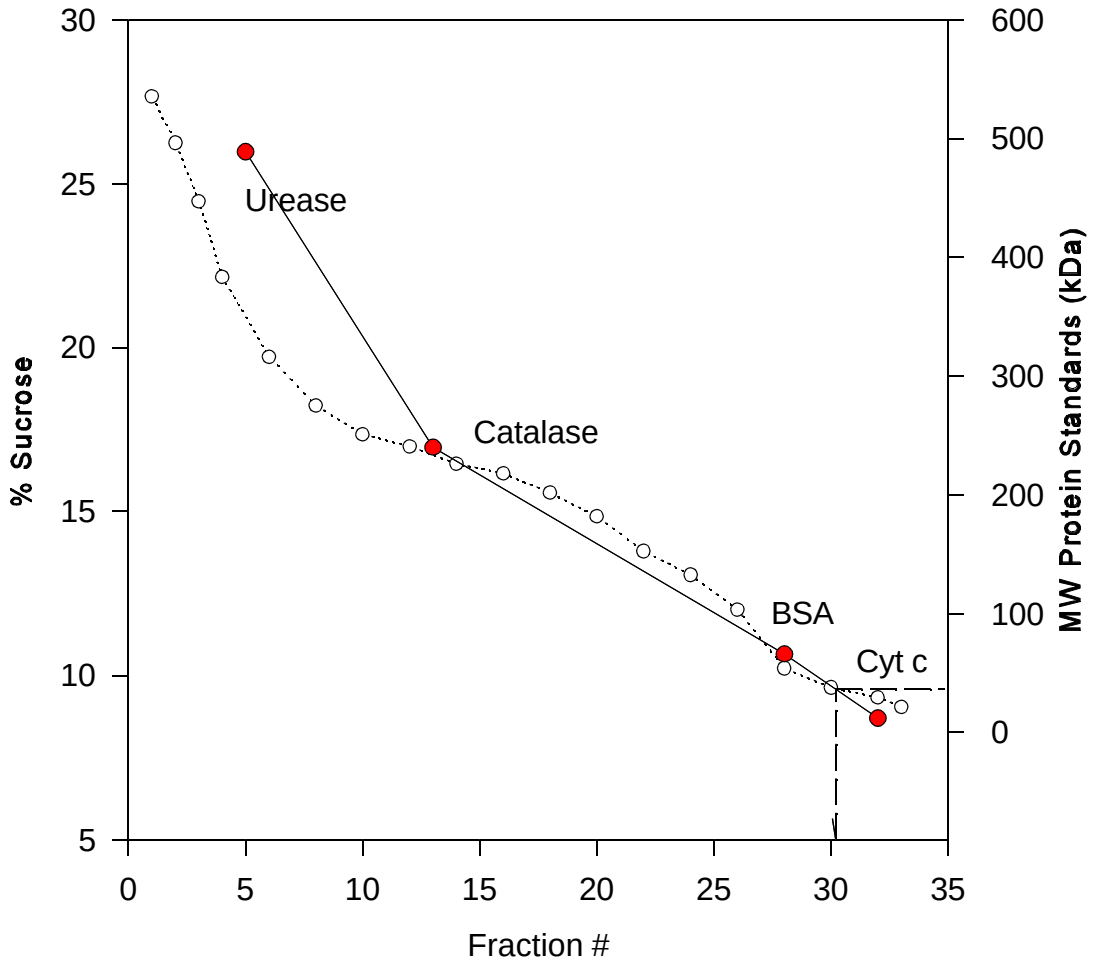

Figure 5.6

Native molecular weight determination of the soluble PTP. The native molecular weight of the phosphatase in the DE52 fraction was determined using sucrose density gradient ultracentrifugation as described under Materials and Methods. The sucrose concentration is indicated by the dashed line connecting the open circles. The positions of the molecular weight markers are represented by the filled circles. The only pNPPase activity observed was present in fraction 31, indicating a molecular weight of ~33kDa.

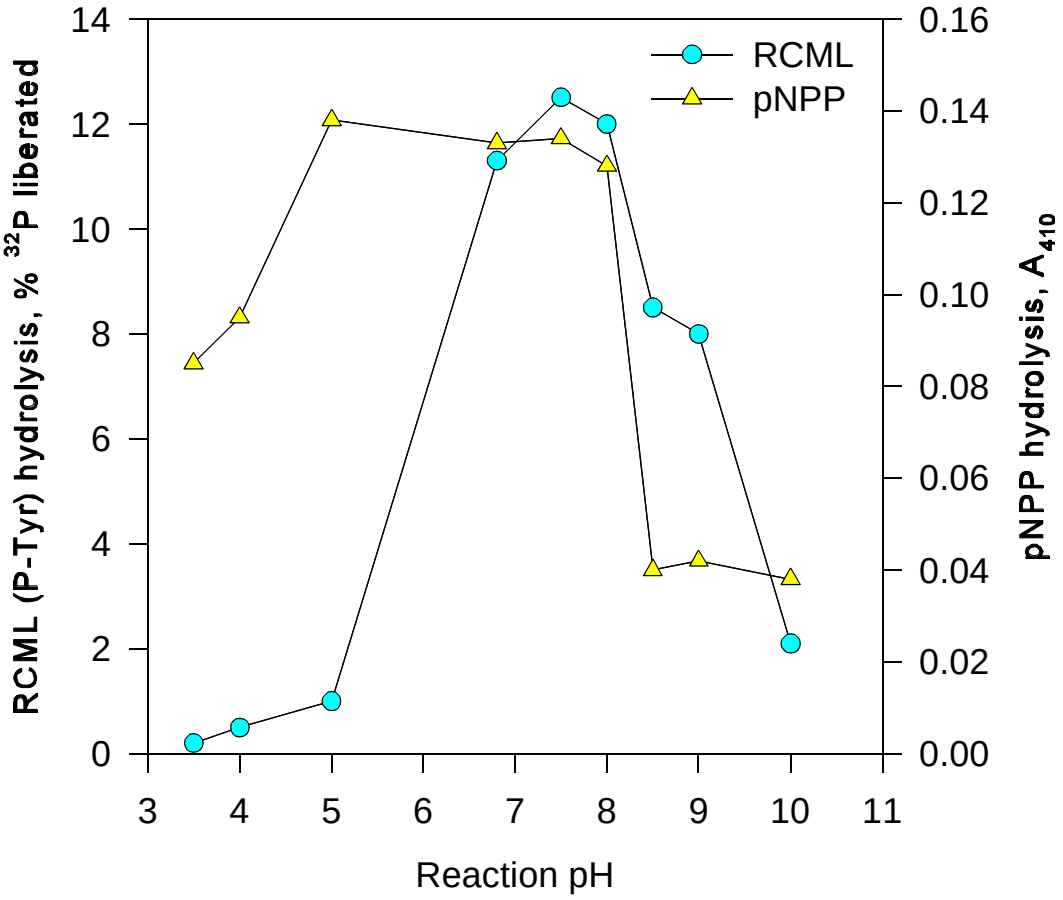

Figure 5.7

Catalytic activity of the soluble phosphatase as a function of pH. Assays of phosphatase activities toward pNPP and RCML (P-Tyr) were performed in 25µL volumes of 40mM buffer, 1mM DTT, 2µM RCML (^{32}P-Tyr), and ~50ng of the DE52 fraction. Hydrolase reactions were performed in 500µL volumes of 40mM buffer containing 1mM DTT, 5mM pNPP, and 10µg of the DE52 fraction. Buffers employed were sodium acetate (pH 3.5, 4.0, or 5.0), 40mM Tris (pH 6.8, 7.5, or 8.0), or 40mM sodium carbonate (pH 8.5, 9.0, or 10.0). Reactions were terminated and products quantified as described under Materials and Methods for the appropriate substrate.

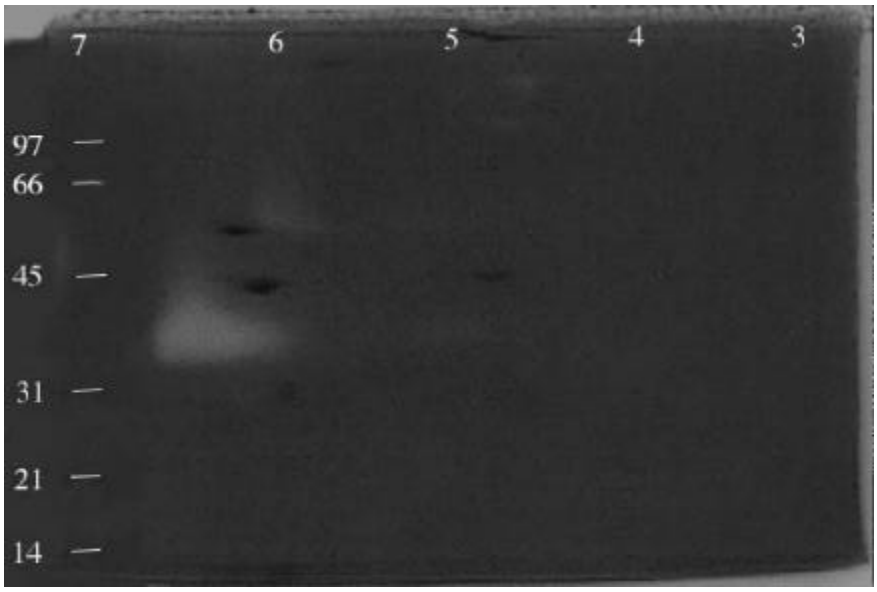

Figure 5.8

Determination of the isoelectric point of the 38kDa PTP. The proteins in the DE52 fraction were resolved in the first dimension by isoelectric focusing in the pH range 3 - 7 (indicated across the top of the gel) and then on a 12.5% SDS gel containing RCML (^{32}P-Tyr) embedded in the gel matrix. Molecular size markers used in the second dimension are shown on the left in kDa.The 38kDa PTP was found to have a p*I* of ~6.5. Shown is the autoradiography film over-laying the dried, coomassie stained gel.

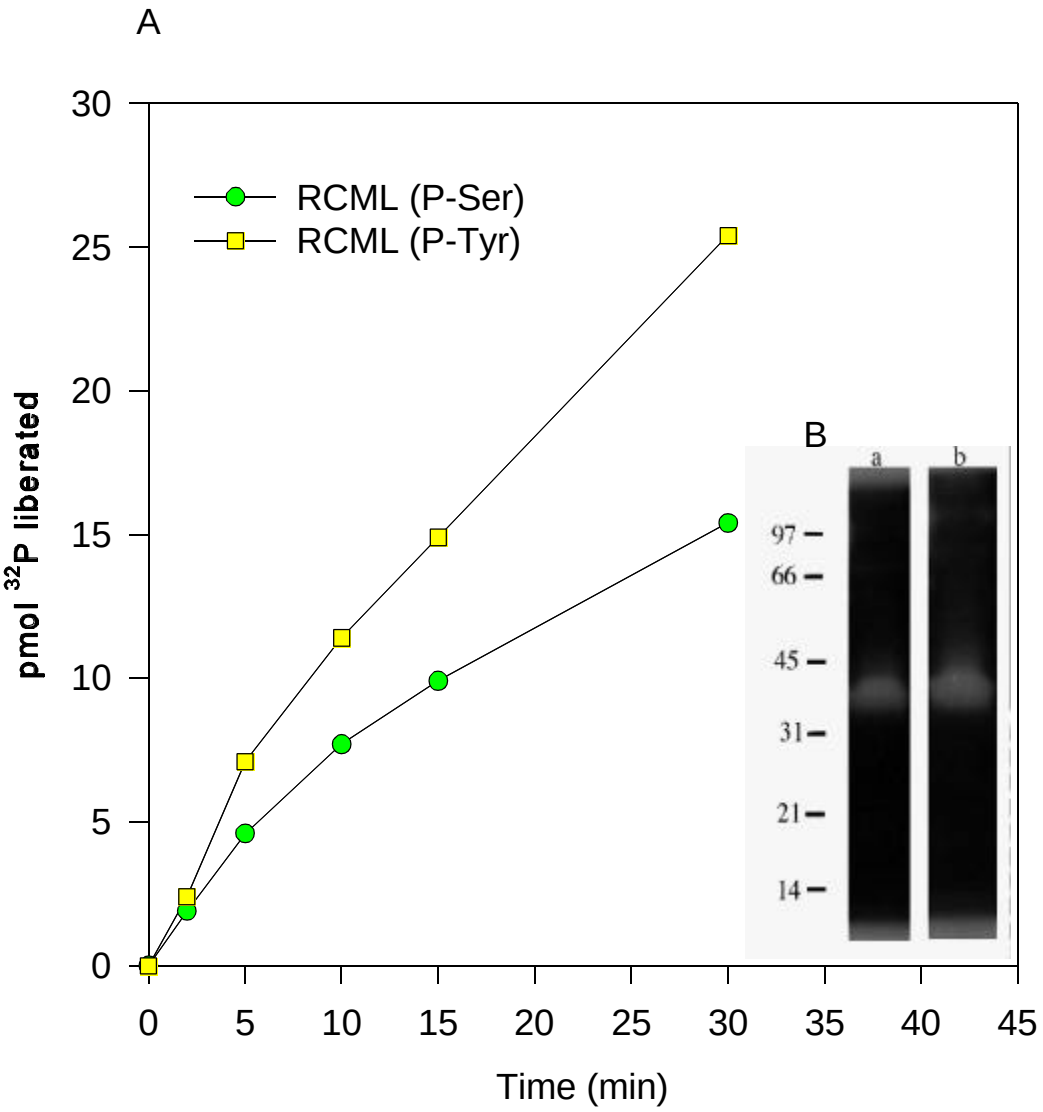

Figure 5.9

RCML (P-Tyr) and RCML (P-Ser) are both dephosphorylated by the DE52 fraction. Panel A, Protein phosphatase reactions performed under standard conditions utilizing 75ng of the DE52 fraction in the presence of either RCML (^{32}P-Tyr) or RCML (^{32}P-Ser) as the substrate, both at 2µM final concentration. Panel B, DE52 fraction (15µg) was applied to a 12.5% polyacrylamide gel containing either RCML (^{32}P-Tyr) embedded in the matrix (lane a) or RCML (^{32}P-Ser) embedd in the matrix (lane b). Molecular weight markers are shown on the left in kilodaltons.

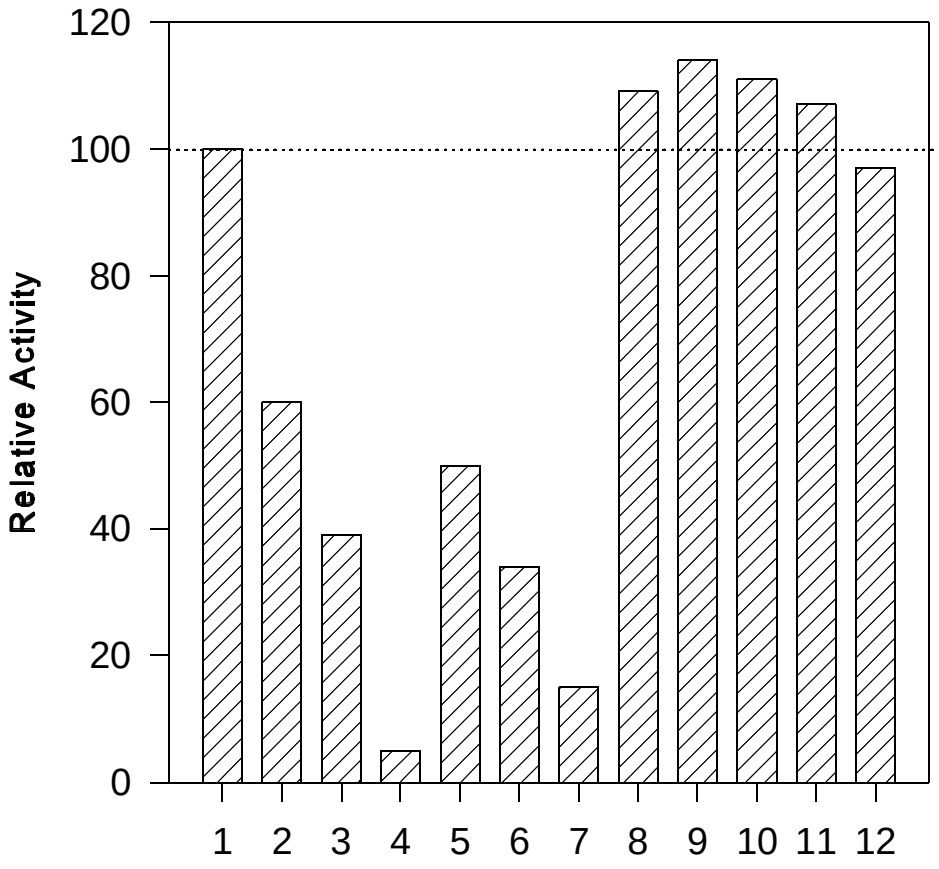

Figure 5.10

The effect known protein phosphatase inhibitors on the soluble DSP. Shown is the PTP activity of the DE52 fraction (~30ng) toward RCML (^{32}P-Tyr) in the presence of well-characterized protein phosphatase inhibitors or sulfydryl-modifying reagents. 1, no effector; 2, 10mM NEM; 3, 10mM HMB; 4, 10mM HMPSA; 5, 10mM IAA; 6, 1mM sodium orthovanadate; 7, 0.1mg/mL heparin; 8, 1mM sodium potassium tartrate; 9, 1mM EGTA; 10, 1mM EDTA; 11, 50µM okadaic acid; 12, 50µM microcystin-LR. To test the effect of protein modifying reagents, the enzyme was assayed following a pre-incubation of 30 minutes at 30°C in NEM, HMB, HMPSA, or IAA at 10mM.

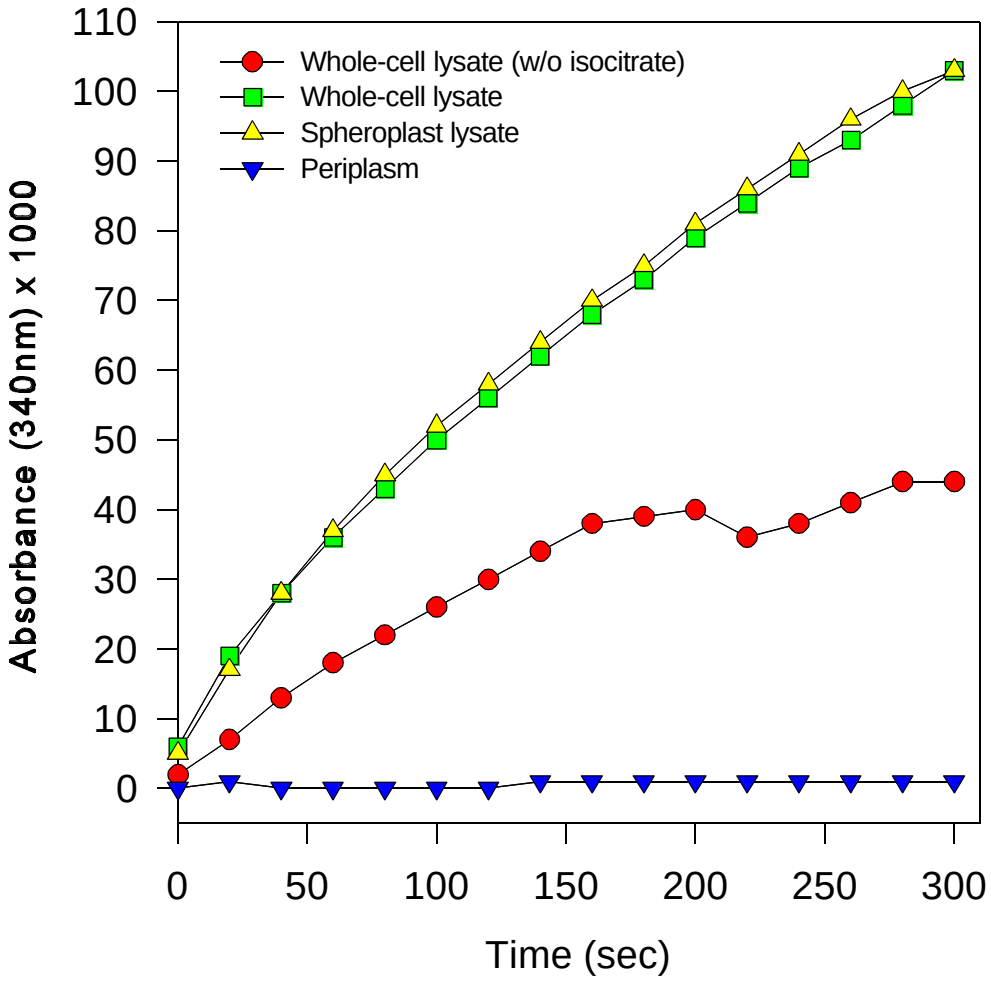

Figure 5.11

Assay of isocitrate dehydrogenase activity in subcellular fractions of *Anabaena*. Aliquots (50µL) of the cell lysate, periplasm, and spheroplast lysate were assayed for isocitrate dehydrogenase (IDH) activity in 1mL volumes containing 1mM isocitrate, 3mM $MgCl_2$, and 0.15mM NAD^+. The formation of NADH was monitored by measuring absorbance at 340nm. Specific IDH activities for the spheroplast lysate and periplasm were $0.1\Delta A_{340}$/min/mg and $<0.005\Delta A_{340}$/min/mg, respectively. The limit of detection was $0.002\Delta A_{340}$/min.

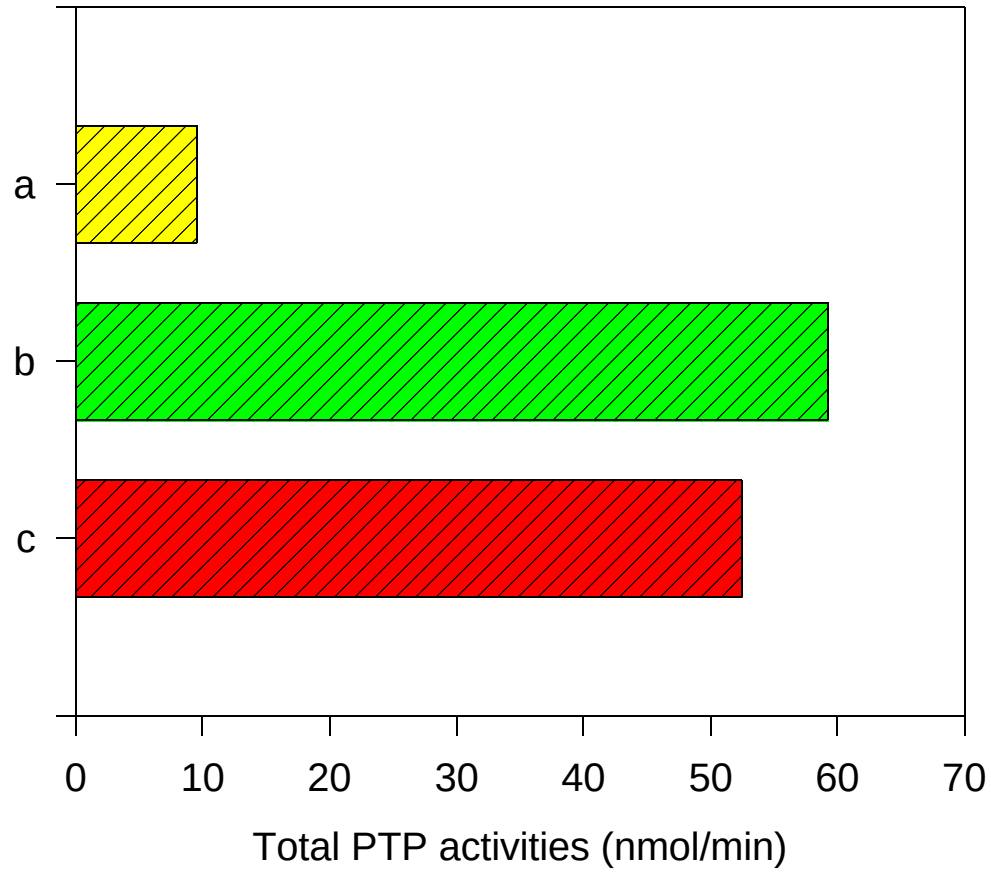

Figure 5.12

Total PTP activities in subcellular fractions of *Anabaena*. Aliquots (1µL) of the spheroplast lysate (a), periplasm (b), and cell lysate (c), prepared as described in the text, were assayed for PTP activity toward RCML (^{32}P-Tyr) at pH 7.5 for 15 minutes at 30°C.

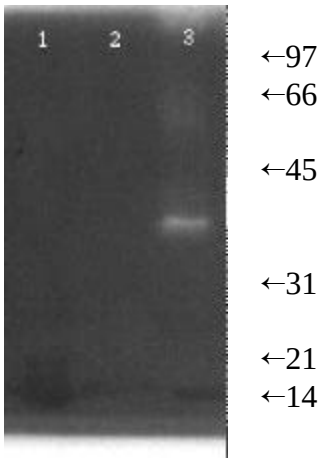

Figure 5.13

In-gel assay of subcellular fractions of *Anabaena*. The PTP activities of subcellular fractions were assayed toward RCML (^{32}P-Tyr) embedded in the matrix of a 12.5% polyacrylamide gel. Lane 1, 500µg of cell lysate; lane 2, 500µg of spheroplast lysate; lane 3, 5µg of periplasm. Molecular weight standards shown on the right in kilodaltons.

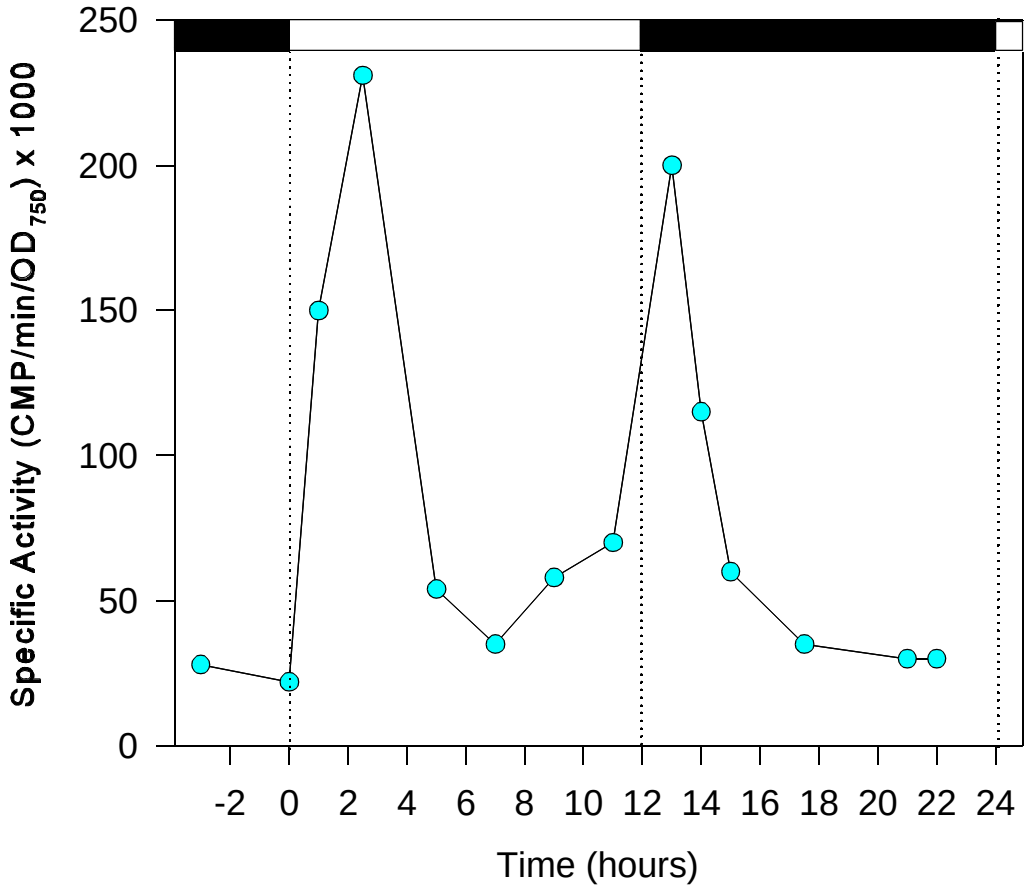

Figure 5.14

PAD activity *in vivo* is rhythmic. Shown is the specific activity of the periplasmic PTP at incremental time points over a 24hr period. The periplasm was harvested from cells obtained from ~60mL of the culture collected every 2 hours. Cells were grown under continuous-culture conditions to maintain a constant culture volume (1.5 liters) and cell density ($OD_{750} \approx 0.5$) as described under Materials and Methods. Ten microliters of the periplasm was assayed phosphatase activity toward RCML (^{32}P-Tyr) under standared conditions as described under Materials and Methods. The solid white bar (top of graph) indicates when the lights were on; the solid black bar indicates when the lights were off.

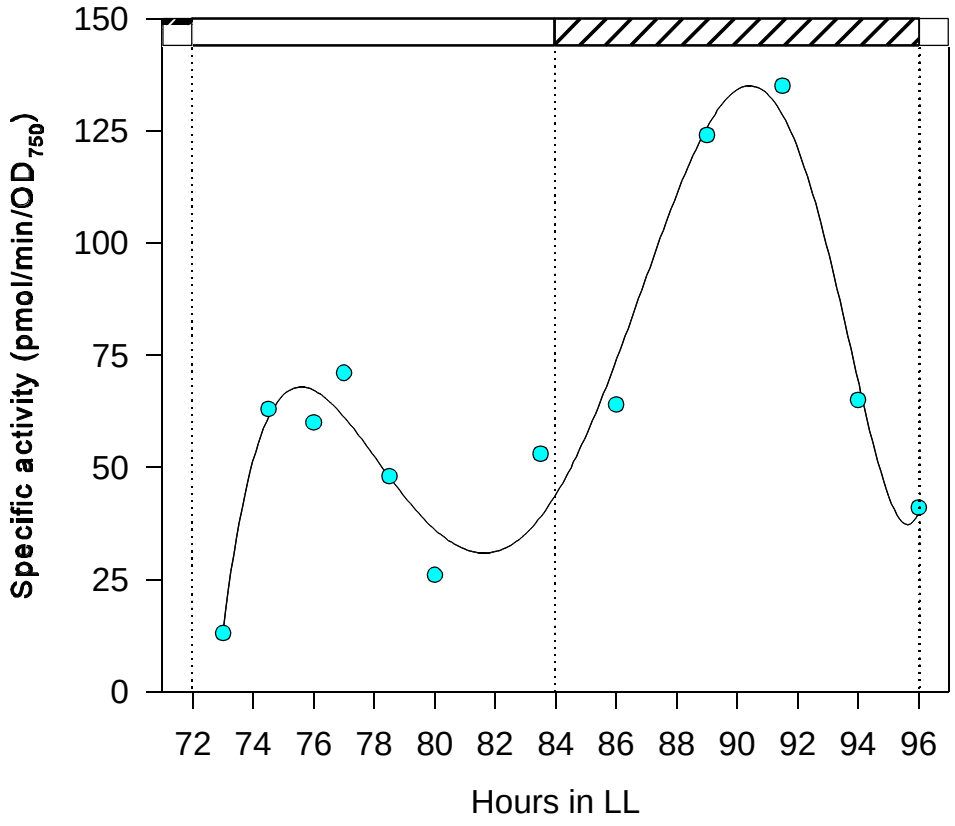

Figure 5.15

Rhythmic PAD activity is independent of light cycle. Shown is the specific periplasmic PTP activity vs time in continuous light (LL). Periplasmic fractions were collected and assayed as described in the figure legend for Figure 5.14. The solid white bar (at top of graph) indicates when the lights would be on, and the hatched bar indicates when the lights would be off, if the light-dark cycle to which the cells were entrained had been continued.

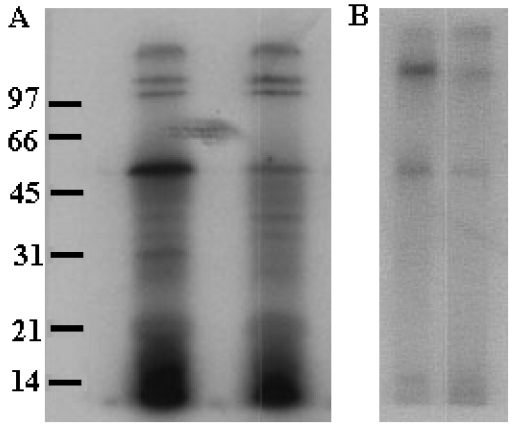

Figure 5.16

Soluble fraction and periplasmic fraction phosphoproteins are dephosphorylated by PAD. A, Soluble fraction proteins (~1mg) were labelled *in vitro* by incubation for 30 minutes at 30°C in 50mM Tris, pH 7.5 containing 0.5mM [γ-^{32} P]-ATP, 0.75mM $MgCl_2$, and 50µM sodium orthovanadate in a total volume of 100µL. Fifty microliters of the labelling reaction was placed on ice and the reaction was terminated by addition of 4x SDS sample solution (5µL). PAD-enriched DE52 fraction (~5µg) was added to the remaining 50µL and incubation was continued at 30°C for an additional 30 minutes. The reaction was terminated by the addition of 5µL of 4x SDS sample solution and 30µL of both samples were applied to a 12.5% polyacrylamide gel. Shown is an autoradiograph of the dried gel: lane 1, radiolabelled proteins; lane 2, radiolabelled proteins following incubation with PAD. B, Periplasmic proteins (~25µg) were labelled and treated with PAD as described above for soluble fraction proteins. Shown is the autoradiograph of the dried gel: lane 1, radiolabelled proteins; lane 2, radiolabelled proteins following incubation with PAD. Molecular weight markers are indicated in kilodaltons.

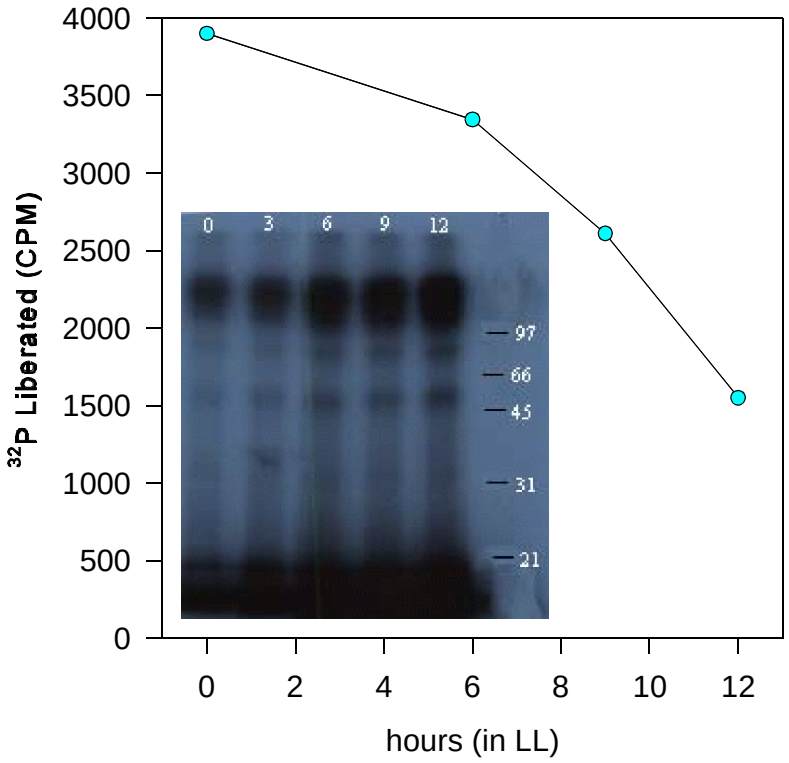

Figure 5.17

Incorporation of ^{32}P into periplasmic phosphoproteins *in vivo* varies inversely with PAD activity. Proteins were labelled *in vivo* by pre-incubating *Anabaena* PCC 7120 entrained to a 12 hour light-dark cycle for 24 hours in the presence of $^{32}PO_4$ (5µCi / mL) in 40ml BG11 media in a 250mL flask. The OD_{750} remained near 0.3 throughout the experiment. The level of ^{32}P incorporated into periplasmic proteins and PAD activity was determined from a 5ml aliquot of the culture taken every 3 hours over a 12 hour period. The periplasmic fraction was prepared by resuspending the cell pellet in 5mL ice-cold 50mM Tris, pH 7.5 containing 1mM DTT and 40% (w/v) sucrose, then resuspending the cells in 1mL 5mM Tris, pH 7.5 containing 1mM DTT. A, PAD activity in 5µg of periplasmic fraction proteins was measured toward RCML (^{32}P-Tyr), assayed under standard conditions (see Materials and Methods). B, Periplasmic proteins (20µg) isolated at each time point were resolved on a 12.5% polyacrylamide gel. Shown is an autoradiograph of dried gel: lane 1, 0 hours; lane 2, 3 hours; lane 3, 6 hours; lane 4, 9 hours; lane 5, 12 hours. Molecular weight markers are indicated in kilodaltons.