CHAPTER 4
DETECTION OF TYROSINE PHOSPHORYLATED PROTEINS IN *ANABAENA* PCC 7120

Objectives:

1. Radiolabel *Anabaena* phosphoproteins *in vitro* using [γ-^{32}P]-ATP for the purposes of:
   a. Detecting tyrosine-[^{32}P]-phosphorylated proteins in *Anabaena* by phosphoamino acid analysis;
   b. Detecting tyrosine-[^{32}P]-phosphorylated proteins by selective dephosphorylation by PTP1B.

Rationale:

As part of his research project, graduate student Bruno McCartney of Malcolm Potts' laboratory demonstrated the presence of phosphotyrosyl-phosphoproteins in *Anabaena* PCC 7120 and other strains of cyanobacteria using commercially-available monoclonal antibodies directed against phosphotyrosine. Cross-reaction between these proteins and the antibodies could be completely eradicated by the addition of free phosphotyrosine, but not phosphothreonine or phosphoserine (1). We asked whether we could detect these phosphotyrosyl-phosphoproteins employing radioisotopic and enzymatic methods to confirm this observation. We sought, therefore, to radiolabel cyanobacterial (i.e., *Anabaena* PCC 7120) proteins *in vitro* and analyze the labelled proteins for [^{32}P]phosphotyrosine content and the ability to act as substrates for the tyrosine-specific protein phosphatase, PTP1B.

Phosphorylation vs Nucleotidylation

Shown in Figure 4.1 are the results of a differential labelling experiment in which cyanobacterial extracts were incubated in the presence of either [γ-^{32}P]-ATP or [α-^{32}P]-ATP. Since no exogenous protein kinases were present, any protein modification event(s) detected must be the result of endogenous enzyme activity(ies). Following incubation of the extracts with ATP, the proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. The gel was then dried and radiolabelled proteins were visualized by autoradiography. Numerous proteins were labelled in the presence of [γ-^{32}P]-ATP. By contrast, none were not labelled in the presence of [α-^{32}P]-ATP. This suggested that the radiolabelled proteins had been modified by phosphorylation and not adenylation. Prominent bands of $M_r \approx$ 21, 27, 52,

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[1]McCartney, B., L. D. Howell, P. J. Kennelly, and M. Potts. (1997). Protein tyrosine phosphorylation in the cyanobacterium *Anabaena* sp. strain PCC 7120. *J. Bacteriol*. **179**: 2314-18.

55, 85, and 100kDa were apparent by autoradiography.

Phosphoamino Acid Analyses of Radiolabelled Phosphoproteins

Phosphoamino acid analyses were performed on several of the radiolabelled proteins following electrophoretic transfer to a PVDF membrane (Fig. 4.2). Analysis of the most prominently-labelled protein band (55kDa) revealed that this protein was phosphorylated exclusively on threonine (Fig. 4.2a). The labelled protein of 27kDa was phosphorylated on serine, threonine, and tyrosine residues (Fig. 4.2b); however, the abundance of phosphotyrosine appeared to be quite low relative to the other phosphoamino acids present. Likewise, the labelled protein of 21kDa was phosphorylated on serine, threonine, and tyrosine (Fig. 4.2c).

Repeated attempts to analyze other phosphoproteins by this method proved unsuccessful. Possible explanations for this include the relatively low quantities of ^{32}P radiolabel incorporated or incorporation of label into acid-labile phosphoamino acids.

Dephosphorylation of Radiolabelled Phosphoproteins by PTP1B

The human protein phosphatase PTP1B displays a restricted specificity for phosphotyrosine (2). We asked, therefore, if any of the radiolabelled cyanobacterial phosphoproteins could be dephosphorylated by PTP1B. In addition, the efficacy of alkaline phosphatase (a non-specific phosphatase) and IphP (a cyanobacterial dual-specificity protein phosphatase) was examined toward these phosphoproteins.

Radiolabelled phosphoproteins were resolved by SDS-PAGE and then transferred to a PVDF membrane as described under Materials and Methods. The PVDF-bound proteins were tested as substrates for the protein phosphatases as described under Materials and Methods. PTP1B liberated a portion of the protein-bound phosphate from four of the five membrane sections examined (Fig. 4.3). This indicated that at least some of the phosphorylated residues were phosphotyrosine. Not surprisingly, given their much broader substrate specificities, alkaline phosphatase and IphP liberated protein-bound phosphate from all of the sections examined and did so in greater quantity than did PTP1B (Fig.4.3). Interestingly, the cyanobacterial protein phosphatase demonstrated the highest level of activity of all the enzymes tested toward most of the sections.

The binding of anti-phosphotyrosine antibodies to cyanobacterial proteins, the liberation of protein-bound phosphate by PTP1B, and the identification of phosphotyrosine in acid hydrolysates of radiolabelled proteins unambiguouly demonstrates the presence of

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[2]Jia, Z., D. Barford, A. J. Flint, and N. K. Tonks. (1995). Structural basis for phosphotyrosine peptide recognition by protein tyrosine phosphatase 1B. *Science* **268**: 1754-58.

tyrosine-phosphorylated proteins, as well as at least one PTK activity, in *Anabaena* PCC 7120.

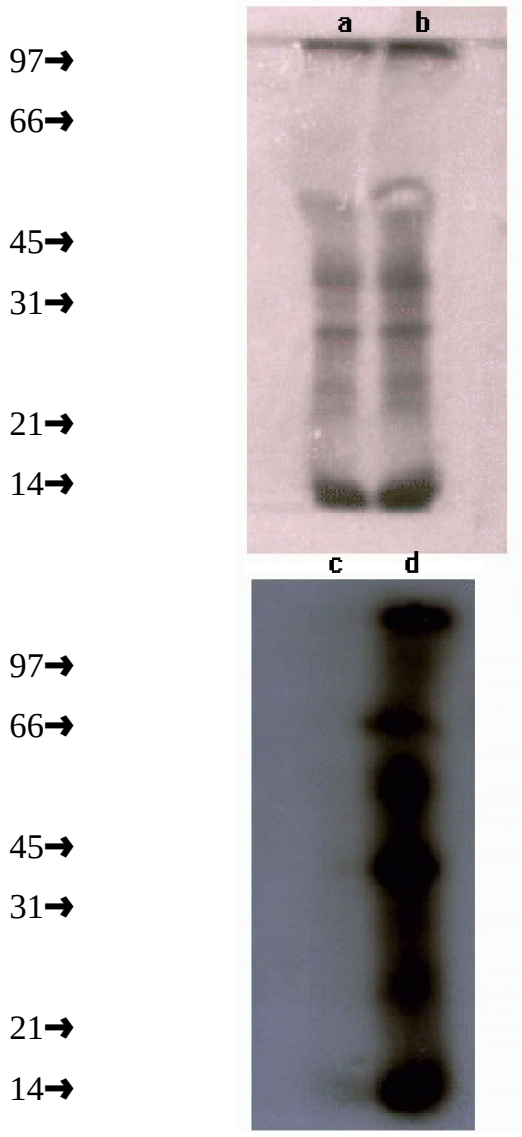

Figure 4.1

Cyanobacterial proteins are phosphorylated by endogenous protein kinases. Top: Shown is the coomassie-stained 12.5% polyacrylamide gel of proteins (~20µg each lane) from *Anabaena* cell extracts following incubation with either (a) [α-^{32}P]-ATP or (b) [γ-^{32}P]-ATP, respectively (see Materials and Methods). Bottom: Shown is an autoradiogram of the same gel. Molecular weight markers are indicated on the left in kilodaltons.

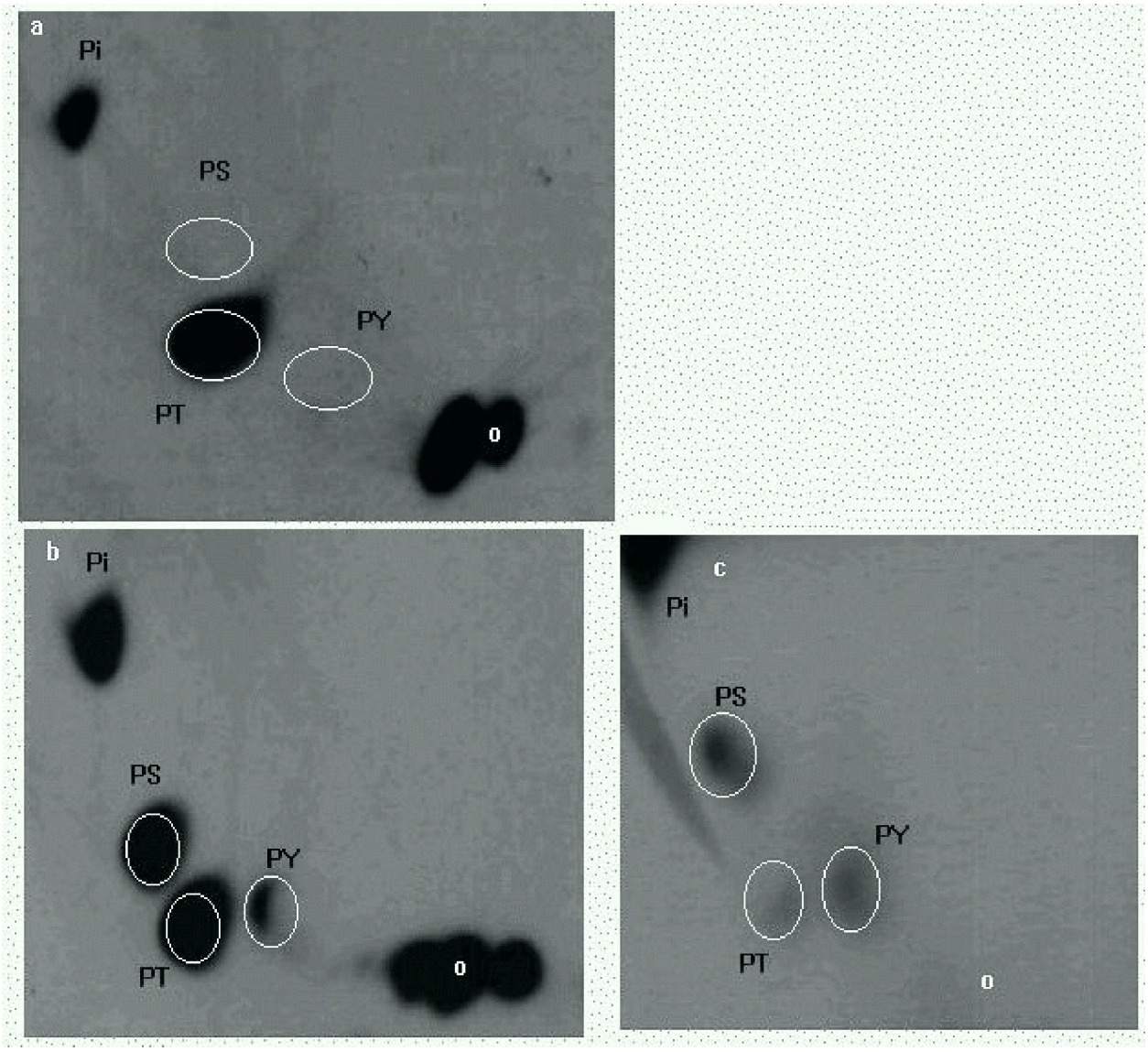

Figure 4.2

Phosphoamino acid analyses of cyanobacterial phosphoproteins. Proteins were radiolabelled, resolved by SDS-PAGE, transferred to a PVDF membrane, and assayed as protein phosphatase substrates as described under Materials and Methods. Panels a, b, and c are the PAA analyses of the 55kDa, 27kDa, and 21kDa proteins, respectively. The locations of phosphoamino acid standards are outlined in white. Pi, inorganic phosphate; PY, phosphotyrosine; PS, phosphoserine; PT, phosphothreonine; o, origin.

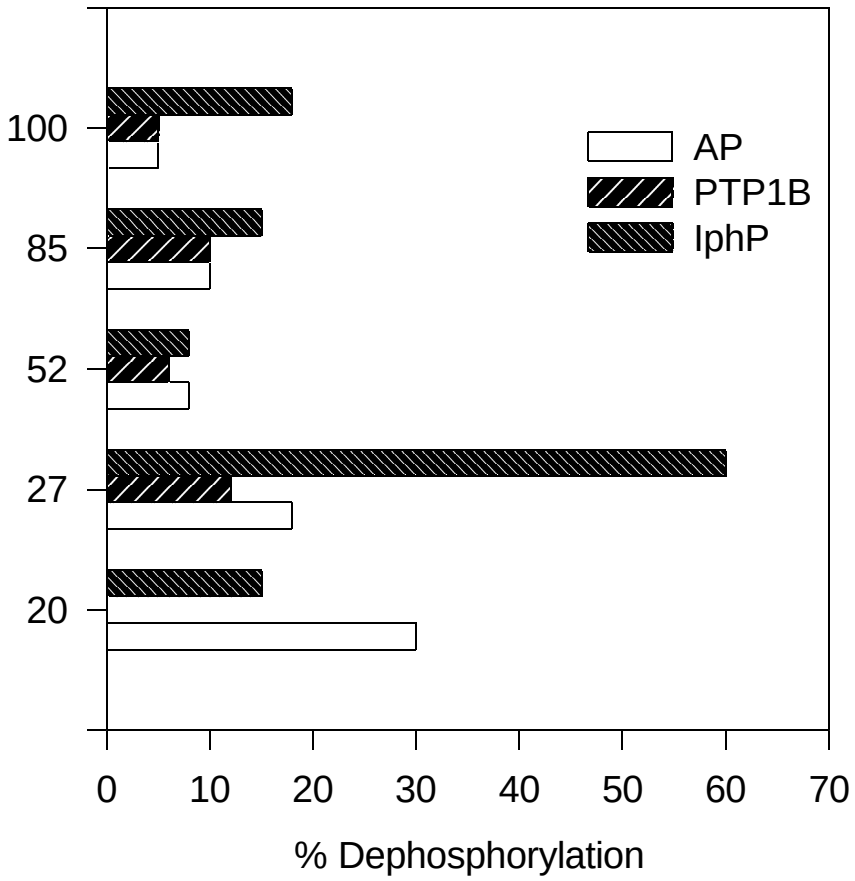

Figure 4.3

PTP1B, IphP, and alkaline phosphatase liberate ^{32}P from radiolabelled *Anabaena* phosphoproteins. PVDF membrane sections containing radiolabelled proteins of the size indicated (in kDa) on the left of the graph were individually incubated in the presence of PTP1B, IphP, or alkaline phosphatase as described under Materials and Methods. Shown is the percentage of membrane-bound ^{32}P released into solution following incubation with the indicated phosphatases.