

CHAPTER 2 MATERIALS & METHODS

Materials

Chemicals purchased were from Sigma or Fisher. Radioisotopes were purchased from DuPont-New England Nuclear. Eco-Lume scintillation fluor was from ICN. Electrophoresis reagents, protein molecular weight- and isoelectric point standards, ampholytes, and mini-protean II apparatus were from Bio-Rad. DE52 cellulose was from Whatman. Cellulose phosphate was from Sigma. Chelating Sepharose and Sephadex G-25 were from Pharmacia. Cellulose TLC plates were from Whatman. Microcystin-LR was from Calbiochem. Okadaic acid was from LC Services Corporation. Heparin-agarose and glutathione-agarose were from Sigma. Phosphatidyl-glycerol, phosphatic acid, cardiolipin, phosphotidylcholine, and phosphotidylinositol were from Sigma. *Nostoc commune* UTEX 584 and DRH1 sheath components were provided by M. Potts. Calf intestinal alkaline phosphatase was from Sigma. Myelin basic protein was from Upstate Biotechnology. Bradford reagent and a standard solution of BSA were from Pierce. Kodak Bio-Max MR X-ray film was from Sigma. Spectrophotometric measurements were made using a Hitachi UV-2000 spectrophotometer. Densitometry measurements were made using a Shimadzu CS9000U scanning densitometer. Scintillation counting was performed using a Beckman LS5800 scintillation counter. A plasmid encoding His-tagged PTP1B was constructed and the protein purified by Ken Bischoff.

Procedures

SDS-PAGE

SDS-polyacrylamide gels were prepared and electrophoresed as described by Laemmli (1). Electrophoresis was performed in a mini-protean II unit (Bio-Rad) at constant 35mA per gel. Samples were prepared by mixing 10-20 μ L of protein sample with 5 μ L of 2x SDS sample solution (2.5% (w/v) SDS, 20% (v/v) glycerol, 0.025% (w/v) bromophenol blue). Samples to be assayed for protein phosphatase activity (see below) were not heated, nor was β -mercaptoethanol added. The gels were stained with Coomassie Brilliant Blue as described by Fairbanks and coworkers (2).

IEF / 2D-PAGE

Isoelectric focusing was performed under non-denaturing conditions (i.e., in the absence of urea). Protein samples (15 μ L) were mixed with 5 μ L 4x Native IEF Sample Buffer (50% (v/v) glycerol, 0.8% (v/v) ampholyte pH 3.5-10, 4% (v/v) ampholyte pH 2.5-5, 0.05% (w/v) bromophenol

¹Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of bacteriophage T4. *Nature* **227**: 680-85.

²Fairbanks, G., T. L. Steck, and D. F. H. Wallach. (1971). Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry*. **10**: 2606-17.

blue) and applied to a 5% acrylamide IEF slab gel. The gel solution was prepared by mixing the following components: 9.7mL water, 2mL acrylamide solution (30% (w/v) acrylamide, 1% (w/v) bis-acrylamide), 48μL pH 3.5-10 ampholyte solution, 240μL pH 2.5-5 ampholyte solution, 50μL 10% (w/v) ammonium persulfate and 20μL TEMED. The gel was cast in a BioRad mini-protean II apparatus. The IEF Protein standards (5μg each) utilized were carbonic anhydrase, pI 6.57, β-lactoglobulin, pI 5.13, and soybean trypsin inhibitor, pI 4.55. Isoelectric focusing was performed for 1 hour at 200V, then 1.5 hours at 400V. Following electrophoretic focusing, the pH gradient was measured by cutting out one lane of the gel and slicing it into ~1cm slices from top to bottom. Each slice was placed into 1mL of 10mM KCl for 15 - 30 minutes and the pH of the liquid measured. The remainder of the gel was stained by soaking in 100mL of 10% (w/v) TCA for 60 minutes, then in 100mL of Fairbanks A stain solution (3) for ~15 minutes. For 2D gels, the lane containing the sample of interest was excised from the unstained gel, equilibrated in SDS-sample buffer (50mM Tris, pH 8.5, 2% (w/v) SDS, 10% (v/v) glycerol, 0.001% (w/v) bromophenol blue) for 15 minutes, and placed across the top of a 12.5% polyacrylamide SDS gel. Electrophoresis of the SDS gel was performed as described above.

Electroblotting to PVDF membranes

Radiolabelled cyanobacterial proteins (~20μg) were resolved by SDS-PAGE on a 12% polyacrylamide gel. During electrophoresis, a PVDF membrane (Millipore) was cut to the same size as the gel and dipped for 2-3 seconds in 100% methanol, then soaked 2-3 minutes in water, and finally soaked at least 15 minutes in transfer buffer (10mM CAPS, pH 11.0, 15% (v/v) methanol). Following electrophoresis, the gel was soaked in 100mL of transfer buffer for 10-15 minutes. The gel and membrane then were sandwiched between two sheets of Whatmann 3MM filter paper that had been cut to size slightly larger than that of the gel and pre-wetted with transfer buffer. The transfer was conducted in a Bio-Rad Trans-Blot SD semi-dry electrophoretic transfer cell for 45 minutes at 12 watts.

Media Formulations

BG11 (ATCC 616): 1.5g NaNO₃, 0.04g 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, 0.222g ZnSO₄ · 7H₂O, 0.39g Na₂MoO₄ · 2H₂O, 0.079g CuSO₄ · 5H₂O, 49.4mg Co(NO₃)₂ · 6H₂O per liter of water), water to 1 liter.

2xYT (ATCC 1320): 16g tryptone, 10g yeast extract, 5g NaCl, water to 1 liter. pH adjusted to 7.0 with NaOH.

³Fairbanks, G., T. L. Steck, and D. F. H. Wallach. (1971). Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry*. **10**: 2606-17.

LB (ATCC 1065): 10g tryptone, 5g yeast extract, 10g NaCl, water to 1 liter. pH adjusted to 7.0 with NaOH.

M9: 12.8g Na₂HPO₄·7H₂O, 3g KH₂PO₄, 0.5g NaCl, 1g NH₄Cl, 2mM MgSO₄, 0.2mM CaCl₂, 20% (w/v) glucose, water to 1 liter.

Growth of Cyanobacteria

Semi-continuous cultures

Anabaena PCC 7120 [ATCC 27893] was cultured photoautotrophically in 10 liters of BG11 media at 25°C. Cultures were grown in a glass 12-liter fermentor surrounded on three sides by fluorescent lights (photon flux density of ~ 500μmol of photons m⁻² s⁻¹) controlled by a timer which provided a 12hr:12hr light / dark cycle. Cells were kept suspended and supplied with atmospheric gases by pumping filter-sterilized air into the media. Unless stated otherwise, cells from 9 liters of the culture were harvested every 7 days in the light-adapted state (6-7 hours into the light cycle). Cells were concentrated and rinsed with fresh BG11 by vacuum filtration. Cell pellets were either lysed immediately for use or stored at -70°C. The culture was then continued by adding fresh BG11 media to a final volume of 10 liters to the remaining cells.

Continuous cultures

Continuous cultures were grown in a "makeshift chemostat" constructed from a 2.8 liter culture flask with a side arm added to maintain the culture volume at 1.5 liters. Cells were initially grown to a density of 0.5 at 750nm in batch culture and then maintained at that density by continuously adding fresh media (BG11) at a flow rate of ~30ml/hr using a peristaltic pump, resulting in a rate of dilution of 30/1500 per hour (0.02 h⁻¹). The culture was illuminated from beneath the flask using a cool-white fluorescent lamp (22 watts). Cells were kept suspended by stirring with a magnetic stir bar and simultaneously bubbling filter-sterile air into the culture. While growing in batch culture, cells were entrained to a 12hr:12hr light:dark cycle and then released into continuous light following the final 12hr dark cycle, at which time continuous dilution also was initiated. Approximately 30ml of eluate was collected each hour and immediately processed for further analyses (e.g., accrual of periplasm).

Preparation of Cyanobacterial Extracts

Cell pellets (typically 10-12g) were resuspended in 3-4 volumes of lysis buffer (50mM Tris, pH 7.5, 1mM EDTA, 1mM EGTA, 1mM DTT, 0.5mM PMSF) supplemented with ~50μg DNase II. Suspended cells were lysed by 3 passes through a French pressure cell (~10k psi). Cell debris was removed by centrifugation at 2500xg for 15 minutes at 4°C. Extracts were stored at 4°C for subsequent analyses.

Expression and Isolation of IphP

E. coli KM001, containing the plasmid pMP005 bearing the *iph* gene (4), were grown with vigorous shaking at 37°C in 250mL of LB media supplemented with 200µg/mL ampicillin until reaching an absorbance of 0.7 at 600nm. Cells were harvested by centrifugation at 4,000xg for 10 minutes at room temperature, and the pellet gently resuspended in 125mL of M9 media that had been pre-warmed to 37°C. The cells were incubated at 37°C for 30 minutes with vigorous shaking (~200 rpm), after which 1M IPTG in water was added to a final concentration of 0.5mM. The cells were incubated 4-6 hours in the presence of IPTG and collected by centrifugation at 4,000xg for 20 minutes at 4°C. The cell pellet was discarded. The supernatant, containing secreted IphP, was supplemented with 2.5mL of a 50x protease inhibitor cocktail (100µg/mL leupeptin, 50mM benzamidine, 500mM EDTA, 25mM DTT) and concentrated to a volume of ~20mL using a stir-cell Amicon concentrator fitted with a YM10 membrane under nitrogen at 45 psi at 4°C. The solution was further concentrated to a final volume of ~2mL using Amicon centrprep 10 centricons, dialyzed against 1x protease inhibitor cocktail containing 10mM sodium acetate, pH 5.0, and stored at 4°C. The protein concentration of the preparation was typically 1-3 mg/mL and IphP represented 50-75% of the protein present as judged by SDS-PAGE.

Expression and purification of p56^{lyn} kinase

E. coli containing a pGEX-KT plasmid encoding a GST/*lyn* kinase fusion protein were the generous gift of Marietta Harrison and Harry Charbonneau of Purdue University. The cells were grown with vigorous shaking at 37°C in 1L of 2xYT media supplemented with 200µg/mL ampicillin until an absorbance of 0.7 at 600nm was reached. Expression of the fusion protein then was induced by the addition of 1M IPTG in water to a final concentration of 0.2mM. The cells were incubated for 3 hours following addition of IPTG and harvested by centrifugation at 4,000xg for 20 minutes at 4°C. Cell pellets were resuspended in 10mL PBS buffer containing 0.1% (w/v) β-mercaptoethanol and the following protease inhibitors: 2mM EDTA, 1mM benzamidine, 0.1 µg/mL aprotinin, 1 µg/mL leupeptin, and 2mM PMSF. Resuspended cells were lysed, on ice, by 5-6 sonication cycles of 70 pulses each at 50% duty cycle, using the small probe and an output setting of 3.5 (sonicator model W185, Heatsystems-Ultrasons, Inc). The samples were cooled on ice for ~1 minute between sonication cycles. Triton X-100 was added to a final concentration of 1% (v/v) following sonication. Cell debris was removed by centrifugation at 10,000xg for 10 minutes at 4°C. The supernatant was mixed with 1mL of a 50% (w/v) slurry of glutathione-agarose beads in PBS and incubated for 30 minutes at 4°C with vertical rotation. The GST/*lyn* kinase-glutathione-agarose beads were washed 4 times (5 minutes of rotation for each wash) in 10mL of PBS containing 1x protease inhibitor cocktail and collected by centrifugation at 1500 x g for 10 minutes at 4°C. The beads then were suspended as a 50% slurry in PBS containin 10% (v/v) glycerol and stored at -20°C. The kinase

⁴Xie, W., B. A. Whitton, J. W. Simon, K. Jager, D. Reed, and M. Potts. (1989). *Nostoc commune* UTEX 584 gene expressing indole phosphate hydrolase activity in *Escherichia coli*. *J. Bacteriol.* **171**: 708-13.

remained active under these conditions for at least 2 years.

The amount of protein associated with the glutathione-agarose beads was determined by adding reduced glutathione (10mM final concentration) to a portion (~10%) of the GST/*lyn*-kinase agarose beads. The mixture was incubated at room temperature for 30 minutes with rotation and the beads were removed by centrifugation. The concentration of protein in the supernatant was assessed using the Bradford protein assay.

Expression and purification of MAP kinase

E. coli BL21(DE3)LysS containing a plasmid encoding histidine-tagged ERK2 was the generous gift of Melanie Cobb. Cell growth, induction of ERK2, and purification were performed as described by Robbins and coworkers (5).

Preparation of ³²P-phosphotyrosyl-RCM-lysozyme

Lysozyme was reduced, carboxymethylated, and maleylated as described by Tonks and coworkers (6). Phosphorylation of the modified protein (RCM-lysozyme) on tyrosine was performed in a 1mL volume of 50mM HEPES, pH 7.5, containing 2mM DTT, 10mM MgCl₂, 0.015% (w/v) Brij-35, 0.5mM [γ -³²P]ATP (10¹⁵ CPM/mol ATP), 1mg RCM-lysozyme, and 20 μ g of GST/*lyn* kinase bound to glutathione-agarose beads. The reaction was performed overnight at room temperature with continuous vertical rotation to ensure thorough mixing of the bead-bound kinase with the soluble components. Following incubation, the kinase-bound beads were removed by centrifugation in a microcentrifuge at 12,000 rpm for 1 minute. The supernatant liquid was collected and phosphotyrosyl-RCM-lysozyme contained therein was purified from other reaction components in the supernatant by TCA precipitation as described (7).

Preparation of ³²P-phosphotyrosyl-casein

Casein (³²P-Tyr) was prepared under identical reaction conditions as for RCM-lysozyme (³²P-Tyr), except that casein (1mg; Sigma) was substituted for RCM-lysozyme. Following removal of GST/*lyn* kinase-agarose beads by centrifugation, phosphotyrosyl-casein was purified from the remaining reaction components by gel filtration chromatography. The supernatant fraction was applied to a column (2x15cm) of Sephadex G-25 that had been equilibrated with 50mM Tris, pH 7.0,

⁵Robbins, D. J., E. Zhen, H. Owaki, C. A. Vanderbilt, D. Ebert, T. D. Geppert, and M. H. Cobb. (1993). Regulation and properties of extracellular signal-regulated protein kinases 1 and 2 *in vitro*. *J. Biol. Chem.* **268**: 5097-106.

⁶Tonks, N. K., C. D. Diltz, and E. H. Fischer. (1988). Purification of the major protein-tyrosine phosphatases of human placenta. *J. Biol. Chem.* **263**: 6722-30.

⁷Tonks, N. K., C. D. Diltz, and E. H. Fischer. (1988). Purification of the major protein-tyrosine phosphatases of human placenta. *J. Biol. Chem.* **263**: 6722-30.

containing 1mM DTT and 0.1mM EGTA. The column was developed with the same buffer. Fractions (1mL) were collected, and 10 μ L portions of each were added to 1mL of scintillation fluor and counted for radioactivity. Fractions containing 32 P-casein (the first peak of radiolabelled material) were stored at -20°C.

Preparation of 32 P-phosphoserine

Casein was phosphorylated on serine using the catalytic subunit of cAMP-dependent protein kinase as described by Kennelly and coworkers (8). Briefly, reactions were carried out overnight at room temperature in 1 mL volumes containing 50mM Tris, pH 7.0, 1mM DTT, 0.5mM [γ - 32 P] ATP (10¹⁵ CPM/mol), 10mM MgCl₂, 0.1mM EGTA, 1mg casein, and ~10 μ g PKA catalytic subunit (Sigma). The lyophilized protein kinase was resuspended in 100 μ L of 50mM Tris, pH 7.0, 1mM DTT and allowed to stand at room temperature for 15 minutes prior to addition of the remaining components of the reaction mixture. The radiolabelled phosphoprotein was purified by passage over a G-25 gel filtration column as described for the preparation of phosphotyrosyl-casein.

Preparation of 32 P-phosphoserine-RCM-lysozyme

RCML (32 P-Ser) was prepared under identical reaction conditions as for casein (32 P-Ser), except that RCM-lysozyme (1mg) was substituted for casein. The radiolabelled phosphoprotein was purified by TCA precipitation as described for the preparation of RCML (32 P-Tyr).

Phosphatase Assays

The activity of protein phosphatases toward 32 P-labelled substrates was generally measured in 25 μ L reaction volumes containing 50mM buffer (sodium acetate, pH 5 or Tris, pH 7.5), 2mM DTT, and 2 μ M substrate-bound [32 P]phosphate. Assays were typically conducted for 30 minutes at 30°C and terminated by the addition of 125 μ L of Stop Solution (0.9M HCl, 90mM Na₄P₂O₇, 2mM NaH₂PO₄, and 4% (w/v) Norit A charcoal) (9). Samples were then mixed briefly on a Vortex mixer and spun in a microcentrifuge for 30 seconds at 12,000 rpm to sediment the charcoal. Seventy-five microliters of the supernatant liquid was then removed, dispersed into 1mL of EcoLume liquid scintillation cocktail, and counted for liberated [32 P]phosphate.

Detection of protein phosphatase activity in polyacrylamide gels was performed essentially

⁸Kennelly, P. J., K. A. Oxenrider, J. Leng, J. S. Cantwell, and N. Zhao. (1993). Identification of a Ser/Thr protein phosphatase from the thermophilic archaeobacterium *Sulfolobus sulfotaricus*. *J. Biol. Chem.* **268**: 6505-10.

⁹Streuli, M., N. X. Krueger, A. Y. M. Tsai, and H. Saito. (1989). A family of receptor-linked protein tyrosine phosphatases in humans and drosophila. *Proc. Natl. Acad. Sci. USA* **86**: 8698-8702.

as described by Gates and coworkers (10). SDS-PAGE was conducted as usual (see above) except that a ^{32}P -labelled protein substrate (e.g. phosphotyrosyl-RCM-lysozyme) was present during the polymerization of the separating gel. This resulted in the covalent attachment of the radiolabelled protein to the gel matrix, thus immobilizing the substrate in the gel. For best results, a total of ~750,000 CPM of protein-bound [^{32}P]phosphate was added to the acrylamide solution regardless of the final concentration of phosphoprotein substrate. Following electrophoresis, the gel was washed twice with gentle shaking for a period of 30 minutes each in a renaturation buffer (50mM Tris, pH 7.5, 0.5mM PMSF, 0.5mM EDTA), then two additional times for a period of 60 minutes each in the same buffer. During the final wash step, DTT was added to a final concentration of 5mM. The gel was then incubated overnight in 50mM Tris, pH 7.5, 0.5mM PMSF, 0.5mM EDTA, and 5mM DTT at room temperature with gentle shaking to allow time for the phosphatase reaction. Following incubation, the gel was stained with Coomassie Brilliant Blue, destained, and dried. The gel was then exposed to X-ray film for 24-48 hours. The presence of protein phosphatase activity was manifested as a clear band against a dark background from the radiolabelled gel, a result of the diffusion of the [^{32}P]phosphate that had been liberated by the hydrolytic action of the phosphatase.

Colorimetric protein phosphatase assays were performed in 100 μL reaction volumes of 100mM buffer (sodium acetate, pH 5.0 for IphP, Tris, pH 7.5 for PAD), containing 1mM DTT, 0.5mM EDTA, 0.5mM EGTA, and 1mM substrate. Following incubation, 50 μL of the reaction mixture was removed and assayed for inorganic phosphate by the malachite green method essentially as described by Lanzetta and coworkers (11). Briefly, Color Reagent was prepared on day of use by mixing 3 volumes of 0.045% (w/v) malachite green solution with 1 volume of 4.2% (w/v) ammonium molybdate solution (4.2% (w/v) ammonium molybdate in 4N HCl). The Color Reagent was allowed to stand at room temperature for at least 20 minutes prior to use. Phosphatase assays were terminated by the addition of 800 μL of Color Reagent, followed by 100 μL of 34% (w/v) sodium citrate. The solution was agitated briefly on a Vortex mixer and allowed to stand at room temperature for ~15 minutes. The absorbance of the solution at 660nm was then measured. The quantity of phosphate was determined from a standard curve prepared using K_2HPO_4 (0 - 10 nmols). Hydrolysis of *p*-nitrophenyl esters was monitored by taking advantage of the absorbance properties of the product *p*-nitrophenol at alkaline pH values ($\lambda_{\text{max}}=410\text{nm}$). Reactions were performed as described above and terminated by adding 400 μL of 0.5M sodium borate, pH 9 and the absorbance of the solution was measured at 410nm.

Dephosphorylation of PVDF-bound phosphoproteins was achieved by first resolving radiolabelled proteins from cell-free extracts of *Anabaena* PCC 7120 by SDS-PAGE and

¹⁰Gates, 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.

¹¹Lanzetta, P. A., L. A. Alvarez, P. S. Reinach, and O. A. Candia. (1979). An improved assay for nanomole amounts of inorganic phosphate. *Anal. Biochem.* **100**: 95-97.

electroblotting the proteins to a PVDF. The radiolabelled proteins were visualized by autoradiography. Membrane sections containing individual bands were excised and cut into portions of equivalent surface area. The portions were incubated in the presence of either PTP1B (0.5µg in 20mM Tris, pH 7.0, 2mM DTT, 5mM EDTA, 0.25M sucrose), IphP (1µg in 50mM sodium acetate, pH 5.0, 2mM DTT), or alkaline phosphatase (AP, Sigma P-7923; 20 units in 50mM Tris, pH 8.2, 2mM DTT) overnight in a 100µL volume at 30 °C. Negative controls substituted the appropriate buffer for the phosphatase. The membrane fragments were removed and the ³²P associated with both the supernatant and membrane fragments were quantified by scintillation counting. Enzymatically hydrolyzed phosphate was defined as the difference in [³²P]phosphate present in samples to which enzyme had been added minus that found in the negative control.

Radiolabelling cyanobacterial phosphoproteins

Proteins (~100µg) in extracts of *Anabaena* PCC 7120 were radiolabelled in 25µL volumes by incubation with 0.2mM α- or γ-[³²P]ATP (~50µCi/µL), 0.5mM MgCl₂, 0.5mM MnCl₂, 0.5mM Na₃VO₄, in 100mM HEPES, pH 7.0 for 2 hours at 30°C. Reactions were terminated by the addition of 4x SDS Sample solution (10µL) and placing on ice.

Phosphoamino acid analysis

Phosphoamino acid analysis was performed to identify the nature of the phosphoamino acids on proteins labelled *in vitro* with [³²P]ATP or *in vivo* with [³²P]P_i. Radiolabelled phosphoproteins were first resolved by SDS-PAGE and then transferred to a PVDF membrane. Individual phosphoproteins were visualized by autoradiography. Membrane sections containing individual radiolabelled proteins were excised and submerged in 100µL of 6N HCl in a 1mL eppendorf tube. The tube was flushed with N₂, sealed, and the sample was heated for 1 hour at 110°C (12). The contents were dried using a speed-vac vacuum dryer (Labconco) with the heater on. The dried membrane was rewetted with 25µL of 100% methanol, immersed in 100µL of water and agitated for ~15 minutes on a Vortex mixer. The membrane was removed with a clean forceps and the free liquid remaining, which contained the free amino acids, was dried using a speed-vac dryer. The dried hydrolysate was dissolved in 5µL of a pH 1.9 buffer (2.2% (v/v) formic acid, 7.8% (v/v) acetic acid) and applied to a cellulose TLC plate as shown in figure 2.1. One microliter of a phosphoamino standard solution, containing 10mM each of phosphotyrosine, phosphothreonine, and phosphoserine, was applied to the TLC plate at the same location as the hydrolysate. Xylene cyanole, 0.5µL of a

¹²Hildebrandt, E., and V. A. Fried. (1989). Phosphoamino acid analysis of protein immobilized on polyvinylidene difluoride membrane. *Anal. Biochem.* **177**: 407-412.

The phosphoamino acid standards were visualized by spraying the plate with a 0.2% (w/v) solution of ninhydrin in acetone followed by a 5 minute incubation at 80°C. Radiolabelled phosphoamino acids derived from the hydrolysate were visualized by autoradiography.

Molybdic acid extraction

The quantification of inorganic [³²P]phosphate was performed essentially as described by Martin and Doty (14). Protein phosphatase assays were performed under standard conditions except that the assays were terminated by the addition ice-cold 100% (w/v) TCA (to a final concentration of 20% (w/v)) rather than Norit A charcoal. An aliquot of the TCA-soluble supernatant, 60μL, was removed to a 1.5mL eppendorf tube. Two-hundred microliters of molybdate reagent (15mg/mL (NH₄)₆Mo₇O₂₄·4H₂O, 25mM H₂SO₄), 10μL of 4mM K₂HPO₄, and 200μL of isobutanol:toluene (1:1) were added. The solution was agitated vigorously on a vortex mixer for 30 seconds, then centrifuged for 3 minutes at 12,000 rpm in a microcentrifuge. An aliquot of the top (organic) layer, 50μL, was mixed with 1mL scintillation fluor and counted for ³²PO₄.

Sucrose density gradient ultracentrifugation

Sucrose density gradients were formed by subjecting 10mL of 15% (w/v) sucrose in the appropriate buffer (see below) to two freeze-thaw cycles in 12mL thin-walled polyallomer tubes (15). Typical gradients ranged from 3 to 30% sucrose.

To detect complex formation between heparin and other proteins, sucrose gradients were formed in 50mM sodium acetate buffer, pH 5.0, containing 2mM DTT. Heparin and the protein in question were then mixed together at a final concentration of 1 mg/mL each in the same buffer, and a 500μL aliquot of the mixture was applied to the top of the sucrose density gradient. The samples were centrifuged in a swinging-bucket rotor (SW-40) for 12 hours at 100,000xg at a temperature of 10°C. Controls contained protein without heparin. Following centrifugation, the tubes were punctured at the bottom and fractions, 500μL, were collected and assayed for protein by the Bradford method (16).

To determine the molecular mass and sedimentation coefficient of PAD, sucrose gradients were formed in 50mM Tris, pH 7.5, containing 1mM DTT. Five-hundred microliters of a protein-standards solution containing 1mg/mL each of urease, catalase, BSA, and cytochrome c was applied to the top one sucrose gradient; 50μL of PAD-enriched DE52 fraction (~35μg) were applied to the

¹⁴Martin, J. B. and D. M. Doty. (1949). Determination of inorganic phosphate. *Anal. Chem.* **21**: 965-67.

¹⁵Baxter-Gabbard, K. L. (1972). *FEBS Lett.* **20**: 117-9.

¹⁶Bradford, M. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-54.

top of another sucrose gradient. The samples were subjected to centrifugation at 100,000 x g for 12 hours at 10°C in a SW-40 swinging-bucket rotor. Following centrifugation, the tubes were punctured at the bottom and 500µL fractions were collected. Aliquots of each fraction from the protein-standards were assayed for urease and catalase activities. Urease was detected by incubating 5µL of 8M urea with a 25µL aliquot of each fraction for 10 minutes, then adding 25µL of Sigma Ammonia Color Reagent and observing precipitation and orange color formation. Catalase was detected by adding 25µL of 3% H₂O₂ to a 25µL aliquot of each fraction and observing bubbling from gas formation. Cytochrome c was located by its absorbance at 417nm; BSA was located by the Bradford protein assay and process of elimination. The PTP was detected in the other sucrose gradient sample by virtue of its pNPPase activity, a common characteristic of PTPs (17). Twenty-five microliters of each fraction was added to 25µL of 50mM Tris, pH 7.5, containing 2mM DTT and 5mM pNPP. The samples were incubated for 1 hour at 30°C and the reactions were terminated by adding 50µL of 0.5M sodium borate, pH 9. Fractions containing a pNPPase activity were identified by yellow color formation.

Heparin-agarose affinity chromatography

A 1mL column of heparin-agarose (Sigma H5380) was equilibrated in 50mM sodium acetate, pH 5.0, containing 2mM DTT. Proteins determined to be dephosphorylated by IphP *in vitro* were each dissolved in a 1mL volume of the same buffer, then applied to the column. The column was washed with 5mL of 50mM sodium acetate, pH 5.0, containing 2mM DTT. Bound proteins were eluted either with 5mL of the same buffer containing 3M NaCl or with 5mL SDS-sample buffer (5% (w/v) SDS, 40% (v/v) glycerol, and 0.05% (w/v) bromophenol blue). Fractions, 1mL each, were collected and analyzed by liquid scintillation counting and / or SDS-PAGE.

¹⁷Chen, L., J. Montserat, D. S. Lawrence, and Z. Zhang. (1996). VHR and PTP1B protein phosphatases exhibit remarkably different active site specificities toward low molecular weight nonpeptidic substrates. *Biochem.* **35**: 9349-54.