Substrate Specificity of IphP, a Cyanobacterial Dual-Specificity Protein Phosphatase with MAP Kinase Phosphatase Activity†

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ABSTRACT: The substrate specificity of the cyanobacterial dual-specificity protein phosphatase, IphP, was explored using a variety of potential substrates. The enzyme displayed phosphomonoesterase activity toward a broad range of peptide, protein, and low molecular weight organophosphate compounds. It displayed little or no hydrolase activity toward phosphodiesters, phosphoramides, carboxyl esters, or sulfoesters. However, it did display measurable pyrophosphatase activity, especially toward ADP and ATP. Among the low molecular weight phosphomonoesters, the presence of an aromatic ring either as part of the leaving group alcohol or immediately adjacent thereto, as in 5′-AMP, was a strong positive determinant for hydrolysis. Among peptide and protein substrates, a rough, but imperfect, correlation between charge character and hydrolysis was noted in which proteins and phosphorylation sites of an acidic nature seemed favored. Heparin affected IphP activity in a substrate-dependent manner. Toward small organophosphates, heparin had no significant effect, but it was inhibitory toward most protein and peptide substrates. However, toward phosphoseryl casein and MAP kinase, it enhanced activity as much as 10-fold. This enhancement was attributed to the ability of heparin to bind to these substrate proteins, as well as IphP, and recruit them to the same microenvironment.

The protein-tyrosine [reviewed in Charbonneau and Tonks (1992) and Walton and Dixon (1993)] and dual-specificity protein phosphatases [reviewed in Keyse (1995)] possess homologous active site sequences (His-Cys-Xaa$_5$-Arg or HAT, for His-Arg-Thiolate) that act via virtually identical catalytic mechanisms to hydrolyze protein phosphomonoesters (Guan & Dixon, 1991a; Zhou et al., 1994). Yet, the protein-tyrosine phosphatases, as their name implies, are highly specific for the hydrolysis of phosphotyrosine residues on proteins while the dual-specificity protein phosphatases act on both the aryl phosphoester phosphotyrosine and the aliphatic phosphoesters phosphoserine and phosphothreonine. Our understanding of the factors responsible for these striking differences in substrate recognition properties remains fragmentary. Data are particularly lacking concerning the dual-specificity protein phosphatases.

The HAT protein phosphatases share the ability to hydrolyze low molecular weight phosphomonoesters such as *p*-nitrophenyl phosphate (pNPP)[1] with relatively high efficiency *in vitro*. While the hydrolysis of such compounds may not be physiologically relevant, it represents a potent tool for studying these enzymes in the laboratory. IphP, the dual-specificity protein phosphatase from the cyanobacterium *Nostoc commune* (Potts et al., 1993), represents the only bacterial dual-specificity protein phosphatase, as well as the only genomically-encoded bacterial enzyme possessing the HAT active site sequence, encountered to date (Kennelly & Potts, 1996). In order to gain insight into the catalytic capabilities and substrate recognition properties of IphP, we surveyed the hydrolytic activity of the enzyme against a range of phosphoproteins, phosphopeptides, low molecular weight organophosphorus compounds, and other organoesters. Herein we report the results of these studies.

EXPERIMENTAL PROCEDURES

Materials. Purchased materials include [γ-^{32}P]ATP (DuPont-New England Nuclear); *p*-nitrophenylthymidine 5-monophosphate, indoxyl 3-sulfate, and indoxyl 3-phosphate (Calbiochem); BCIP (Lambda Fluorescence Co.); ADP (Boehringer-Mannheim); nitrophenyl acetate (Aldrich); Bio-Rex AG 1-X8 ion exchange resin (Bio-Rad); alkaline phosphatase (Worthington); and chelating Sepharose fast flow (Pharmacia). Heparin, heparin−agarose, chondroitin sulfate, poly[Glu$_4$:Tyr], poly(L-lysine), poly(L-histidine), poly(L-aspartate), sodium vanadate, the catalytic subunit of the cyclic AMP-dependent protein kinase, *N*-ethylmaleimide, glutathione−agarose, and all low molecular weight phosphomonoester, phosphodiester, sulfoester, pyrophosphate, and carboxyl ester substrates not listed previously were from Sigma. All buffers and routine lab chemicals not listed above were from Fisher.

Routine Procedures. Protein concentrations were measured by the method of Bradford (1976) using premixed reagent and a protein standard solution from Pierce (Rockford, IL). SDS−polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). Phosphoamino acid analysis was performed as described by Cooper et al. (1983).

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[1] Abbreviations: pNPP, *p*-nitrophenyl phosphate; MBP, myelin basic protein; MAP kinase, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; RCM-lysozyme, reduced, carboxamidomethylated and maleylated lysozyme; R_{II}, regulatory subunit type II of the cAMP-dependent protein kinase; cAPK, cAMP-dependent protein kinase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; SDS−PAGE, SDS−polyacrylamide gel electrophoresis; YINAS peptide, Glu-Asn-Asp-Tyr-Ile-Asn-Ala-Ser-Leu; PTP, protein-tyrosine phosphatase.

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Expression and Isolation of IphP, Lyn Kinase, and MAP Kinase. IphP was expressed in *E. coli* and isolated as described in Potts et al. (1993). The material obtained represents an approximately equimolar mixture of IphP and β-lactamase. *E. coli* cells containing plasmid pGEX-KT plasmid, which encodes the catalytic domain of the lyn kinase as a glutathione-*S*-transferase fusion protein, were the generous gift of Profs. Marietta Harrison and Harry Charbonneau of Purdue University. Growth of *E. coli*, induction of the expression of plasmid-encoded genes, and absorption of the GST/lyn kinase fusion protein onto glutathione−agarose were performed essentially as described by Guan and Dixon (1991b). The protein was then used to phosphorylate protein and peptide substrates while immobilized on the glutathione−agarose beads. *E. coli* cells containing a plasmid designed to express MAP kinase (a.k.a. ERK2) with His_6 at its amino terminus were the generous gift of Prof. Melanie Cobb of the University of Texas Southwestern Medical Center. These cells were grown, MAP kinase expression was induced, and the protein kinase was isolated by chromatography on a nickel-chelate resin as described by Robbins et al. (1993).

Preparation of ^{32}P-Labeled Phosphoprotein and Phosphopeptide Substrates. Lysozyme was reduced, carboxymethylated, and maleylated by the procedure of Crestfield et al. (1963) as modified by Tonks et al. (1988) to give RCM-lysozyme. Casein, histone H2a, histone H2b, RCM-lysozyme, and myelin basic protein were phosphorylated on serine using the catalytic subunit of the cAMP-dependent protein kinase and [γ-^{32}P]ATP [$(1-10) \times 10^{14}$ cpm/mol] using the procedure described in Kennelly et al. (1993) for preparing phosphoserylcasein. Following their phosphorylation, these proteins were separated from unreacted ATP etc. by gel filtration chromatogrphy over Sephadex G-25 (Kennelly et al., 1993). The sole exception was RCM-lysozyme, which was isolated by TCA precipitation as described by Tonks et al. (1988). Kemptide was phosphorylated under similar conditions, then purified free of unreacted [^{32}P]ATP, P_i, etc. by passage through a 1 mL column of Dowex AG1X8 equilibrated in 30% acetic acid, and then lyophilized and redissolved in 5 mM HEPES, pH 7.0.

Casein, MBP, poly(Glu_4:Tyr), RCM-lysozyme, YINAS peptide (Daum et al., 1993), and Val^5-angiotensin II were phosphorylated on tyrosine using a p56 lyn kinase−GST fusion protein and [γ-^{32}P]ATP [$(1-10) \times 10^{14}$ cpm/mol]. Reactions were performed in a volume of 1 mL of 50 mM HEPES, pH 7.5., containing 2 mM DTT, 10 mM $MgCl_2$, 0.015% Brij-35, 5.25 mM [γ-^{32}P]ATP, 80 nmol of protein or peptide, and glutathione−agarose beads containing 20 μg of bound p56 lyn kinase−GST fusion protein. Incubation was carried out at room temperature, overnight, on a rotating platform to ensure thorough mixing of soluble components with the agarose-immobilized protein kinase. The agarose beads were then pelleted by centrifugation. Casein, MBP, and poly(Glu_4:Tyr) were separated from unreacted ATP etc. by gel filtration chromatography over Sephadex G-25 as described above. P-Tyr RCM-lysozyme was isolated by TCA precipitation as described above. YINAS peptide and Val^5-angiotensin II were purified free of unreacted [^{32}P]ATP, P_i, etc. by passage through a 1 mL column of Dowex AG1X8 equilibrated in 30% acetic acid and then lyophilized and redissolved in 5 mM HEPES, pH 7.0.

The R_{II} subunit of cAPK, 70 μg, was phosphorylated on serine using 0.2 μg of casein kinase II by incubation overnight at 30 °C in 50 mM Tris, pH 7.5, containing 150 mM NaCl, 10 mM $MgCl_2$, and 1 mM ATP. The volume was 250 μL. Unreacted ATP, P_i, etc. were then removed from the phosphorylated protein by dialysis versus 5 mM Tris, pH 7.5, containing 100 mM NaCl and 10 mM $MgCl_2$.

MAP kinase, 50 nmol, was phosphorylated on threonine and tyrosine by incubation overnight at 30 °C in a volume of 1 mL of 50 mM Tris-HCl, pH 7.0, containing 15 mM $Mg(OAc)_2$, 5.25 mM ATP, and 3.75 μg of MEK. Phosphorylated MAP kinase was then separated from unreacted ATP, P_i, etc. by dialysis versus 50 mM Tris-HCl, pH 7.5, containing 2 mM DTT, 0.1 mM EGTA, and 50 μM benzamidine. The phosphoamino acid composition of MAP kinase was checked by the procedure of Cooper et al. (1983).

Preparation of Phosphopolylysine and 3-Phosphopolyhistidine. Partially phosphorylated histidine and lysine homopolymers were prepared as described in Wong et al. (1994).

Assay of IphP Activities. Phosphatase activity against ^{32}P-labeled proteins, polypeptides, and peptides was measured as follows. IphP, 10−800 ng of protein (depending upon the avidity of the substrate in question), was incubated at 30 °C for 30 min in a volume of 25 μL containing 50 mM sodium acetate, pH 5.0, 2 mM DTT, and 2 μM substrate-bound phosphate. Reactions were terminated by the addition of 125 μL of 0.9 M HCl containing 90 mM $Na_4P_2O_7$, 2 mM NaH_2PO_4, and 4% (w/v) Norit A charcoal. Following vigorous agitation on a Vortex mixer, the sample was centrifuged on a microfuge for 30 s at 12000*g* to sediment the charcoal, and a 75 μL sample of the supernatant liquid was removed, dispersed into 1 mL of EcoLume liquid scintillation cocktail (Westchem), and counted for released [^{32}P]phosphate.

Phosphohydrolase activity toward nonradioactive, low molecular weight phosphomonoesters and pyrophosphates was measured as follows. IphP (0.3 μg, 1.5 μg, or 7.5 μg) was incubated at 30 °C for 30 min in a volume of 100 μL of 100 mM sodium acetate, pH 5.0, containing 1 mM DTT, 0.5 mM EDTA, and 1 mM substrate. Following incubation, a 50 μL aliquot of the reaction mixture was removed and assayed for inorganic phosphate by the malachite green method as described by Lanzetta et al. (1979), with the exception that the Sterox was omitted. Hydrolysis of *p*-nitrophenyl esters was measured under identical conditions. However, at the end of the 30 min incubation period, the reaction was terminated by adding 400 μL of 0.5 M sodium borate, pH 9.0, shaking vigorously, and determining the quantity of *p*-nitrophenolate present by measuring the optical density of the mixture at 410 nm. For esters of 5-bromo-4-chloro-3-hydroxyindole and 3-hydroxyindole, which produce an insoluble blue chromophore upon hydrolysis, reaction conditions identical to those described above were used. The time it took to produce a blue color similar to that observed with the standard substrates BCIP or indoxyl 3-phosphate, respectively, was taken to be inversely proportional to the relative activity of the enzyme. This figure was then normalized to the activity of IphP toward the standard substrate, pNPP, by multiplying it times the activity of the enzyme toward BCIP or indoxyl 3-phosphate relative to the pNPP as measured using the quantitative malachite green technique.

Cyclic phosphodiesters were assayed using a two-stage incubation procedure. In the first stage, IphP, 5.0 μg, was incubated with the diester in exactly the same manner as described above for phosphomonoesters except that the total volume was decreased from 100 to 50 μL. At the end of the 60 min incubation, reaction was halted by adding 5 μL of 1M sodium borate buffer, pH 9.0 (IphP generally displays little or no activity at alkaline pH's). In order to hydrolyze any phosphomonoesters created, alkaline phosphatase, 10 μg, was then added and the mixture incubated at 30 °C overnight. Controls contained either no IphP or no alkaline phosphatase. At the end of this time, the quantity of inorganic phosphate present was measured using malachite green.

Affinity Chromatography on Heparin−Agarose. A 1 mL column of heparin−agarose (Sigma H5380) was equilibrated in 50 mM sodium acetate, pH 5.0, containing 2 mM DTT. Samples of IphP substrate molecules was dissolved in a 1.0 mL volume of the same buffer and applied to the column. The column first was washed with 5 mL of 50 mM sodium acetate, pH 5.0, containing 2 mM DTT; then bound proteins were eluted either with 5 mL of the same buffer with 3 M NaCl added or with 5 mL of SDS-sample buffer [a solution of 5% (w/v) SDS containing 40% (v/v) glycerol and 0.05% bromophenol blue]. Fractions, 1 mL each, were collected and analyzed by liquid scintillation counting and/or SDS−PAGE.

Sucrose Density Gradient Ultracentrifugation. Sucrose density gradients were formed by subjecting 10 mL of 50 mM sodium acetate, pH 5.0, containing 2 mM DTT and 10% (w/v) sucrose to two freeze−thaw cycles in 12 mL thin-walled polyallomer tubes (Baxter-Gabbard, 1972). Typical gradients ranged from 3 to 30% sucrose. To detect complex formation between heparin and other proteins, heparin and the protein in question were mixed together at a final concentration of 1 mg/mL each in 50 mM sodium acetate, pH 5.0, containing 2 mM DTT. A 500 μL aliquot of this mixture was then applied to the top of the sucrose density gradient and centrifuged in a swinging-bucket rotor for 12 h at 35000*g* at a temperature of 10 °C. Controls contained protein without heparin. Following centrifugation, the tubes were punctured at the bottom, and fractions, 500 μL, were collected and assayed for protein by the Bradford method (Bradford, 1976).

RESULTS

What Range of Hydrolytic Reactions Can IphP Catalyze? Like all phosphatases containing the HAT active site motif, IphP displays significant catalytic activity toward low molecular weight phosphomonoesters such as BCIP (Xie et al., 1989). Therefore, recombinant IphP was challenged with a broad range of low molecular weight phosphomonoesters, phosphodiesters, and related compounds in order to assess its catalytic capabilities as well as to obtain clues as to the structural features that it recognizes in substrates. As can be seen from the data in Table 1, IphP exhibited detectable activity toward a broad range of phosphomonoesters. Phosphomonoesters in which the phosphate group was esterified to an aromatic ring, such as pNPP, α- and β-naphthyl phosphate, BCIP, and phosphotyrosine, consistently proved to be among the compounds most rapidly dephosphorylated. Nucleotide monophosphates also were readily dephosphorylated, as well as thiamin phosphate and pyridoxal phosphate, while most sugar phosphates generally were hydrolyzed at rates only a few percent of that observed with pNPP. Phospholipids proved to be very poor substrates, and the free phosphoamino acids phosphoserine and phosphothreonine proved even less efficacious, being dephosphorylated at barely detectable rates. No activity could be detected toward any of the phosphoramides (phosphoarginine, phosphopolylysine, or 3-phosphopolyhistidine), sulfate esters (indoxyl 3-sulfate, 5-bromo-4-chloro-3-indolyl sulfate, or *p*-nitrophenyl sulfate), or carboxyl esters (5-bromo-4-chloro-3-indolyl butyrate or 5-bromo-4-chloro-3-indolyl caprylate) tested with the exception of *p*-nitrophenyl sulfate, which was hydrolyzed at a low, but detectable rate: 0.3% that with pNPP. Low but detectable phosphodiesterase activity, 0.1% that displayed against pNPP, could also be detected against 3′,5′-cAMP, but not against bis(*p*-nitrophenyl) phosphate or *p*-nitrophenylthymidine 5′-monophosphate. Somewhat surprisingly, hydrolysis of pyrophosphate bonds could be detected at rates that, in the case of ADP, approached those seen with some of the better phosphomonoester substrates. Pyrophosphatase activity was sensitive to vanadate or to pretreatment of the enzyme with *N*-ethylmaleimide (data not shown), both of which had been previously demonstrated to inhibit IphP (Potts et al., 1993). Analysis of ADP by ion-exchange chromatography indicated that it contained no detectable AMP (data not shown), while incubations were performed in which as much as 30% of the ADP was hydrolyzed, indicating that the phosphate produced was not derived from a phosphomonoester contaminant.

Does IphP Discriminate between Potential Phosphopeptide and Phosphoprotein Substrates? The activity of IphP toward a number of exogenous protein and peptide substrates was determined. As can be seen in Table 2, IphP dephosphorylated a number of proteins and peptides that were phosphorylated on serine, threonine, and/or tyrosine residues, including casein, RCM-lysozyme, poly(Glu_4:Tyr), MBP, MAP kinase, YINAS peptide, and Val^5-angiotensin II. No activity could be detected toward histone H2a, histone H2b, or kemptide. With regard to MAP kinase, which was phosphorylated on both threonine and tyrosine, IphP was able to completely dephosphorylate both of these amino acid residues (Figure 1, $t = 30$ min). Analysis of the phosphoamino acid content of MAP kinase when it had been only partially dephosphorylated revealed, however, that while the quantity of phosphotyrosine remaining was only 55% of its initial value, over 90% of the phosphothreonine remained, indicating that the phosphotyrosine was by far the more rapidly dephosphorylated of the two (Figure 1, $t = 5$ min).

Heparin Enhances the Rate of Dephosphorylation of Phosphoserylcasein and MAP Kinase by IphP. Heparin inhibits a broad spectrum of HAT protein phosphatases (Daum et al., 1993). However, when IphP was assayed in the presence of heparin, its effect proved highly substrate-dependent. With phosphotyrosylcasein, phosphoseryl or phosphotyrosyl RCM-lysozyme, phosphoseryl/phosphothreonyl or phosphotyrosyl MBP, or YINAS peptide, significant inhibition was observed (Table 2). However, with pNPP, BCIP, and Val^5-angiotensin II, little or no inhibition was apparent, while with phosphoserylcasein or MAP kinase a severalfold enhancement in the rate of dephosphorylation was observed (Table 2) that, in the presence of 0.1 mg/mL heparin, ranged from 4- to 10-fold.

Table 1: Relative Activity of IphP toward Low Molecular Weight Organophosphorus Compounds and Other Esters[a]

substrate class	substrate name	relative activity (% pNPP)
phosphomonoesters	*p*-nitrophenyl phosphate (pNPP)	100.0
	phosphoserine	0.1
	phosphothreonine	0.2
	phosphotyrosine	26.7
	5-bromo-4-chloro-3-indolyl phosphate (BCIP)	25.0
	indoxyl 3-phosphate	38.2
	α-naphthyl phosphate	100.0
	β-naphthyl phosphate	50.0
	4-methylumbelliferyl phosphate	79.2
	glucose 6-phosphate	8.3
	glucose 1-phosphate	5.0
	sucrose 6′-monophosphate	3.7
	ribose 5-phosphate	17.7
	5′-AMP	40.4
	3′-AMP	36.2
	2′-AMP	21.0
	5′-dAMP	16.5
	2′-dAMP	28.3
	5′-GMP	12.6
	5′-dGMP	12.1
	5′-UMP	33.0
	5′-CMP	21.0
	5′-IMP	42.0
	inositol 2-phosphate	0.4
	phytic acid	1.7
	β-glycerol phosphate	5.5
	O-phosphorylethanolamine	0.2
	phosphorylcholine	0.3
	phosphatidic acid	1.8
	NADP	3.8
	thiamin phosphate	16.4
	pyridoxal phosphate	44.0
pyrophosphates	sodium PP_i	0.9
	sodium triphosphate	0.5
	sodium tetraphosphate	0.7
	ADP	12.6
	ATP	1.9
	thiamin pyrophosphate	0.7

[a] The compounds listed were tested as possible substrates for the hydrolase activity of IphP as described under Experimental Procedures. All substrates were present at a final concentration of 1 mM. Activity is reported relative to that observed with a standard substrate, pNPP, which was set equal to 100%. The lower limit of detection for these assays was estimated to be 0.1% the activity observed toward pNPP.

What Is the Mechanism by Which Heparin Enhances the Dephosphorylation of Phosphoserylcasein and MAP Kinase? Activation by heparin was concentration-dependent (Figure 2). Dephosphorylation of MAP kinase was enhanced at much lower concentrations of heparin than proved the case with phosphoserylcasein. Both curves could be shifted to the right by addition of unphosphorylated substrate protein (data not shown). This suggests that the differences observed reflect, at least in part, the fact that the MAP kinase preparation was phosphorylated to a nearly stoichiometric level, while in the phosphoserylcasein preparation only about 1 casein molecule in 10 contained [^{32}P]phosphate. For both proteins, maximal enhancement could be reached using 0.1 mg/mL heparin. This concentration therefore was chosen as the standard for subsequent experiments.

Salts such as sodium chloride, sodium sulfate, and sodium phosphate either had no effect on the dephosphorylation of phosphoserylcasein and MAP kinase or proved inhibitory. Since this suggested that the polyanionic nature of heparin might be responsible for its enhancing effects, a variety of other polyanions were tested for their ability to act as heparin mimetics. Phospholipid vesicles comprised of phosphatidylserine, phosphatidylcholine, phosphatidylinositol, or cardiolipin had little or no effect, while phosphatidylglycerol and phosphatidic acid enhanced dephosphorylation of phosphoserylcasein 2.6- and 2.0-fold, respectively. The latter had little or no effect on MAP kinase dephosphorylation, however. Both poly(aspartic acid) and single-stranded DNA were mild, 2.0−2.5-fold, stimulators of IphP activity toward both substrates, while tRNA was without effect. Chondroitin sulfate enhanced MAP kinase dephosphorylation nearly 3-fold, with little or no effect on phosphoserylcasein dephosphorylation.

Kinetic analyses revealed that the addition of 0.1 mg/mL heparin decreased the apparent K_m of IphP for MAP kinase by about 4-fold, but had no significant effect on k_{cat} (Table 3). [Because the cAMP-dependent protein kinase phosphorylates casein to a very low stoichiometry (McGowan & Cohen, 1988), the K_m of phosphoserylcasein proved too high to permit accurate determination of its kinetic parameters.] Since this suggested that heparin may act by increasing the affinity of IphP for MAP kinase and phosphoserylcasein, it was asked whether these and other potential substrates could bind heparin. Using a heparin−agarose affinity column, it was determined that IphP binds heparin, as does casein, phosphoserylcasein, phosphorylated MAP kinase, and phosphotyrosyl-Val^5-angiotensin II (data not shown). Formation of IphP−heparin, casein−heparin, and MAP kinase−heparin complexes in free solution also was observed using sucrose density gradient centrifugation (data not shown). The

Table 2: Relative Activity of IphP toward Peptide and Protein Substrates[a]

substrate (phosphoamino acid)	enzyme activity ($pmol^{-1}$ min^{-1} mg^{-1})	effect of heparin (% activity w/ o heparin)
casein (P-Ser)	40	910
casein (P-Tyr)	7000	10
RCM-lysozyme (P-Ser)	5050	29
RCM-lysozyme (P-Tyr)	245	13
poly(Glu_4:Tyr) (P-Tyr)	117	39
MAP kinase (P-Thr and P-Tyr)	3667	650
myelin basic protein (P-Ser and P-Thr)	2083	9
myelin basic protein (P-Tyr)	3450	1
R_{II} subunit of cAPK (P-Ser)	1250	5
histone H2a (P-Ser)	nd[b]	
histone H2b (P-Ser)	nd	
YINAS peptide (P-Tyr)	520	13
Val^5-angiotensin II (P-Tyr)	111	76
kemptide (P-Ser)	nd	
BCIP	3.91×10^6	80
pNPP	3.75×10^6	100

[a] The listed phosphoprotein, phosphopolypeptide, and phosphopeptide substrates were tested as potential substrates for IphP as described under Experimental Procedures in the presence and absence of 0.1 mg/mL heparin. All substrates were present at a concentration of 2 μM substrate-bound phosphate with the exception of BCIP and pNPP, which were present at 2 mM. [b] nd = not detectable; less than 10 pmol min^{-1} mg^{-1}.

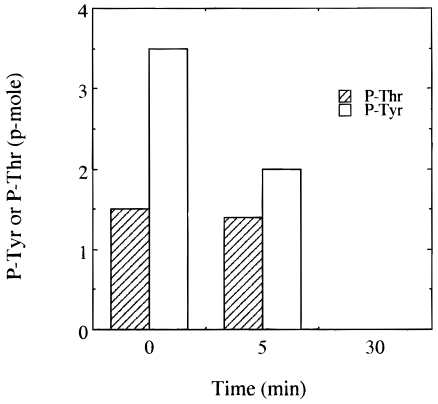

FIGURE 1: IphP dephosphorylates the phosphotyrosine on MAP kinase much more quickly than phosphothreonine. Shown are the quantities of phosphotyrosine (open bars) and phosphothreonine (hatched bars) recovered upon phosphoamino analysis of [^{32}P]MAP kinase prior to incubation with IphP (t = 0), and following incubation with IphP under standards conditions for periods of 5 and 30 min.

following passed through the heparin−agarose column without binding: phosphotyrosylcasein, MBP, phosphotyrosyl MBP, phosphoseryl or phosphotyrosyl RCM-lysozyme, phosphorylated poly(Glu_4:Tyr), YINAS peptide, and pNPP. With the exception of phosphotyrosyl-Val^5-angiotensin II, whose dephosphorylation was slightly inhibited by heparin, the ability of a potential substrate to bind heparin and the enhancement of its dephosphorylation by IphP directly correlated.

Next, it was asked whether proteins that would be expected to have a high affinity for heparin, i.e., those that would bear a significant net positive charge at pH 5.0, could interfere with its ability to enhance the dephosphorylation of phosphoserylcasein or MAP kinase by IphP. As can be seen in Figure 3, addition of a 10-fold weight excess, relative to heparin, of proteins with p*I*'s of 7.0 or greater resulted in

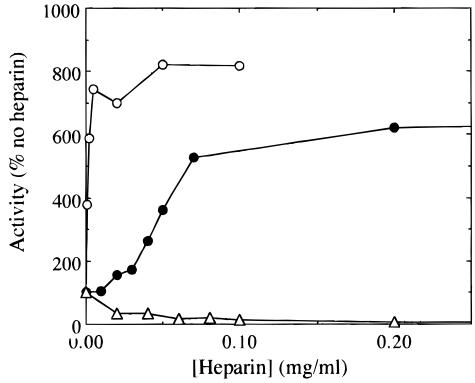

FIGURE 2: Enhancement of IphP-catalyzed MAP kinase and phosphoserylcasein dephosphorylation by heparin is concentration-dependent. The activity of IphP toward [^{32}P]MAP kinase (○), [^{32}P]-phosphoserylcasein (●), and [^{32}P]phosphotyrosyl RCM-lysozyme (△) was measured under standard conditions in the presence of the indicated concentrations of heparin. Activity is reported relative to that observed in the absence of heparin, which was set equal to 100%.

Table 3: Kinetic Parameters for Dephosphorylation of Selected Substrates by IphP[a]

substrate	K_m (mM)	k_{cat} (s^{-1})	$10^{-6} \times$ k_{cat}/K_m (s^{-1} M^{-1})
pNPP	0.05	315	6.3
BCIP	0.50	110	0.22
phosphotyrosine	0.56	115	0.21
5′-AMP	0.71	1100	1.55
ribose-5-P	0.77	150	0.19
glucose-6-P	0.25	80	0.32
indoxyl-3-P	0.21	145	0.69
MAP kinase (−heparin)	0.32	400	1.25
MAP kinase (+heparin)	0.081	300	3.75
RCM-lysozyme (P-Tyr)	0.009	10.5	1.12
RCM-lysozyme (P-Ser)	0.041	167	4.07
YINAS peptide (P-Tyr)	0.050	24	0.48

[a] Listed are the V_{max} and k_{cat} of IphP toward selected substrates from those listed in Tables 1 and 2. All assays were performed under standard conditions with the exception of MAP kinase, which was assayed in both the absence and presence of 0.1 mg/mL heparin.

complete blockage of heparin's enhancing effects. Proteins with p*I*'s below 7.0 were either only partially effective or, in the case of those with p*I*'s of 5.0 and below, completely impotent.

DISCUSSION

IphP was challenged *in vitro* with a wide range of potential substrates. Relative to its phosphomonoesterase activity toward pNPP, IphP exhibited little or no ability to hydrolyze phosphodiesters, sulfoesters, phosphoramides, or carboxyl esters. It did, however, exhibit detectable pyrophosphatase activity. In the case of ADP, this activity was quite pronounced, approaching within an order of magnitude that observed with the most efficacious phosphomonoesters. Overall, however, the pattern observed was more consistent with that predicted for a phosphomonoesterase than for a general-purpose esterase.

Although IphP displayed detectable levels of phosphomonoesterase activity toward a wide range of compounds, patterns of preference/selectivity were manifest. The presence of an aromatic ring provided a strong positive determinant. While in the case of phosphotyrosine, BCIP, pNPP, etc. it might be argued that this merely reflects the greater

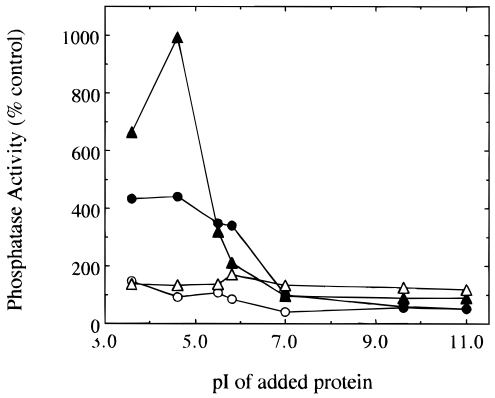

FIGURE 3: Effect of added proteins on heparin enhancement of phosphoserylcasein and MAP kinase dephosphorylation by IphP. IphP activity toward phosphoserylcasein and MAP kinase was measured under standard conditions, in the presence and absence of 0.1 mg/mL heparin, with the exception that the following proteins were added to a final concentration of 1 mg/mL: amyloglucosidase (p*I* 3.6), ovalbumin (p*I* 4.6), bovine serum albumin (p*I* 5.5), catalase (p*I* 5.8), hemoglobin (p*I* 7.0), cytochrome *c* (p*I* 9.6), and lysozyme (p*I* 11.0). Shown is the activity relative to assays in the absence of either added heparin or added proteins versus the p*I* of the protein added. Symbols used included (○) casein as substrate in the absence of heparin, (●) casein as substrate in the presence of heparin, (△) MAP kinase as substrate in the absence of heparin, and (▲) MAP kinase as substrate in the presence of heparin. In the presence of heparin, but in the absence of added proteins, phosphocasein phosphatase activity was 430% that observed without heparin while MAP kinase phosphatase activity was 655% that observed without heparin.

leaving group potential of an aromatic alcohol, this cannot account for the differences observed between the rates of hydrolysis of sugar phosphates such as ribose 5-phosphate and nucleotide monophosphates such as 5′-AMP. For both of these compounds, the leaving group is the 5-OH of ribose, whose leaving group potential should be little affected by the addition of the adenine ring. Hints of a similar propensity of aromatic groups to enhance the dephosphorylation of alkyl phosphates were noted by Zhang in studies of the protein-tyrosine phosphatases Yop51 and PTP1 (Zhang, 1995), while the X-ray structure of complexes of the protein-tyrosine phosphatase PTP-1B with a peptide substrate revealed the presence of a hydrophobic pocket that interacts with a leucine residue at the $P+1$ position (Jia et al., 1995). The parallel with the more fragmentary pattern observed with pyrophosphates is also noteworthy.

With regard to protein and peptide substrates, IphP was both selective and highly efficient. The k_{cat}/K_m values for the peptide and protein substrates YINAS, MAP kinase, and phosphoseryl and phosphotyrosyl RCM-lysozyme were comparable to those of the most efficacious low molecular weight organophosphorus compounds, pNPP and 5′-AMP (Table 3). Moreover, the enzyme's K_m values for phosphopeptides and proteins were generally an order of magnitude lower than those for naturally occurring organophosphates. IphP's k_{cat}/K_m values toward protein and peptide substrates also exceeded, by roughly 10−100-fold, the highest k_{cat}/K_m value, 3.2×10^4 M^{-1} s^{-1}, observed for the dual-specificity protein phosphatase VHR toward peptide substrates derived from MAP and JNK kinase (Denu et al., 1995). The contrast in the ability of IphP to very efficiently hydrolyze the phosphoryl groups of phosphothreonine and phosphoserine when found in the context of a protein or peptide as opposed to when they are present as the free phosphoamino acids, as well as the lesser contrasts seen with sugar versus nucleotide phosphates, is suggestive of a phosphohydrolase intended to tackle large, macromolecular substrates.

IphP also displayed selectivity among protein and peptide substrates. Kemptide, histone H2a, histone H2b, and, in an earlier study (Potts et al., 1993), glycogen phosphorylase all proved highly refractory to dephosphorylation by IphP. The mechanisms underlying this discrimination are not clear. Histone's H2a and H2b are highly basic proteins, and kemptide contains only basic and neutral amino acid residues, while casein, RCM-lysozyme, poly(Glu_4:Tyr), and the R_{II} subunit of cAPK are all quite acidic, suggesting at first glance that the gross charge of macromolecular substrates may play a significant role. However, MBP is also extremely basic, yet it was readily dephosphorylated by IphP. The marked difference between the rates at which phosphotyrosylcasein was dephosphorylated versus the phosphoseryl form, 175-fold, and the 21-fold difference in the rates of dephosphorylation of phosphoseryl versus phosphotyrosyl RCM-lysozyme, indicates that the character of the site at which proteins are phosphorylated may also affect their interaction with IphP.

The behavior of IphP toward MAP kinase, as well as phosphoserylcasein, provided a further suggestion of the possible role of charge character in the recognition of large, macromolecular substrates. Although heparin is widely regarded as a general protein-tyrosine phosphatase inhibitor (Daum et al., 1993), toward IphP its effects were dramatically substrate-dependent. While significant inhibition was observed with substrates such as phosphotyrosylcasein, phosphotyrosyl or phosphoseryl RCM-lysozyme, poly(Glu_4:Tyr), phosphoseryl/phosphothreonyl and phosphotyrosyl MBP, and phosphotyrosyl-YINAS peptide, heparin produced a many-fold enhancement in the rate of dephosphorylation of phosphoserylcasein and MAP kinase. Since heparin had little effect on the dephosphorylation of low molecular weight substrates such as pNPP and BCIP, the observed perturbations cannot reflect either direct binding of heparin to the active site cleft or an alteration in the intrinsic catalytic efficiency of the enzyme. Rather, these seemingly disparate effects of heparin appear to have been mediated through its ability to influence the association of macromolecular substrates with IphP.

The evidence for this associative model for the enhancement of the dephosphorylation of MAP kinase and phosphoserylcasein is 3-fold. First, IphP and all of the phosphoproteins whose dephosphorylation was enhanced by heparin also bind heparin. Second, proteins that would be expected to bind heparin tightly, and thus block the binding of IphP and/or substrates, blocked heparin stimulation. Third, the kinetic effects of heparin on the dephosphorylation of MAP kinase, a severalfold decrease in K_m with little effect on V_{max}, are consistent with heparin acting to recruit MAP kinase or phosphoserylcasein to the same microenvironment as IphP. Heparin's inhibitory effects toward other macromolecular substrates presumably result from blockage of the latter's binding to IphP without providing a compensatory recruitment mechanism. A similar associative mechanism has been proposed to explain the stimulatory effects of another polyanionic species, phospholipid vesicles, on the dephosphorylation of phosphotyrosyl MBP and MAP kinase by the protein-tyrosine phosphatase PTPIC (Zhao et al., 1993).

This represents the most extensive study of the substrate specificity of a dual-specificity protein phosphatase conducted to date, yet much remains to be answered. Based on the patterns observed in this study, it would appear that IphP can interact with potential substrates at three "sites". First, there is the active site pocket itself. Next, there appears to be a hydrophobic interaction site adjacent to the active site pocket that interacts with aromatic groups directly proximal to the phosphoester itself, such as the nucleotide bases of 5′-AMP or ADP, or the heterocyclic aromatic ring of pyridoxal phosphate. Lastly, there appears to be a more distal site in which charge character dominates. In exceptional instances, heparin can interact with this last site and bridge IphP with substrates that interact more avidly with heparin than they do with IphP.

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