CHAPTER 3
SUBSTRATE SPECIFICITY & CATALYTIC PROPERTIES OF IPHP

Objectives:

The physiological role of IphP in *Nostoc commune* UTEX 584 has not yet been determined. In this series of experiments we sought to ascertain the most likely role of the phosphatase *in vivo* by exploring its substrate specificity and catalytic abilities *in vitro*. Specifically, the objectives were to

1. Determine if IphP displays selectivity among potential protein / peptide substrates;
2. Determine and compare kinetic parameters for macromolecular vs low molecular weight substrates of IphP;
3. Determine the mechanism of substrate-specific heparin enhancement.

Rationale:

Since IphP had been observed in initial studies to hydrolyze both the phosphoryl groups on the proteins RCM-lysozyme and casein, as well as low molecuar weight phosphomonoesters such as pNPP and 5-bromo-4-chloro-3-indolyl phosphate, it possessed the catalytic potential to function *in vivo* either as a regulatory enzyme (DSP) or a nutritional enzyme (phosphate scavenger). We sought to determine the most plausible role for the phosphatase *in vivo* by challenging it *in vitro* with a wide range of low-molecular weight phosphomonoesters, phosphopeptides, and phosphoproteins, asking whether it displayed preferences for any particular group. Our working hypothesis was that an enzyme involved in the scavenging of phosphorus would be expected to readily hydrolyze a diverse assortment of low molecular weight- and protein substrates, showing little preference or specificity, while a PTP with regulatory functions would most likely display a preference for proteins over low molecular weight phosphoesters, as well as a degree of selectivity among potential protein substrates.

Relative IphP Activity Toward Potential Peptide & Protein Substrates

IphP displays selectivity among potential peptide and protein substrates

IphP dephosphorylated a number of peptides and proteins that had been phosphorylated on serine, threonine, and / or tyrosine residues (Table 3.1). The enzyme displayed significant protein phosphatase activity toward casein (^{32}P-Ser), casein (^{32}P-Tyr), RCML (^{32}P-Ser), RCML (^{32}P-Tyr), poly (Glu_4Tyr) (^{32}P-Tyr), MAPK (^{32}P-Thr / ^{32}P-Tyr), myelin basic protein (^{32}P-Ser / ^{32}P-Thr), the regulatory subunit of cAMP-dependent protein

kinase (^{32}P-Ser), and the peptides YINAS (^{32}P-Ser)(1) and angiotensin II (^{32}P-Tyr)(2). In contrast, no activity could be detected toward histone H2a (^{32}P-Ser), histone H2b (^{32}P-Ser), polyphosphohistidine, polyphospholysine,or the peptide kemptide (^{32}P-Ser)(3). IphP thus discriminates among phosphoprotein and phosphopeptide substrates *in vitro*.

IphP favors protein/peptide substrates vs low MW organophosphates in vitro

The results of kinetics analyses performed for selected IphP substrates are listed in Table 3.2. These experiments revealed a propensity by IphP to hydrolyze protein and peptide substrates over low molecular weight organophosphate compounds. Overall, the K_M values for protein and peptide substrates were about 10-fold lower than those obtained with low molecular weight compounds. Likewise, the k_{cat}/K_M values for protein and peptide substrates were, in general, higher than those obtained for low molecular weight organophosphates, especially those most likely to be encountered by IphP in nature.

IphP dephosphorylates P-Tyr of MAPK faster than P-Thr

In mammalian cells, the protein kinase MAPK is phosphorylated on the tyrosine and threonine residues in the sequence Tyr-X-Thr (4). This dual phosphorylation event activates the enzyme. IphP dephosphorylated both the phosphotyrosine and the phosphothreonine residues on MAP kinase (^{32}P-Tyr/^{32}P-Thr). However, following incubation with IphP for various time periods, acid hydrolysis of MAPK (^{32}P-Tyr / ^{32}P-Thr) revealed that the phosphotyrosine residue was dephosphorylated at a significantly faster rate than the phosphothreonine residue (Fig. 3.1). Roughly 50% of the phosphotyrosine residues were dephosphorylated during the initial 5 minutes of the reaction, while over 90% of the phosphothreonine residues remained. Within 30 minutes, however, both residues were completely dephosphorylated (Fig. 3.1).

Heparin Affects IphP Activity in a Substrate-Specific Manner

During the course of these studies it was discovered that heparin, a known inhibitor of most protein tyrosine phosphatases, could enhance IphP activity toward our standard substrate, casein (^{32}P-Ser). Further studies revealed that the stimulatory effect was strikingly

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[1]amino acid sequence: ENDY(P)INASL

[2]amino acid sequence: DRVY(P)VHPF

[3]amino acid sequence: LRRAS(P)LG

[4]Nishida, E., and Y. Gotoh. (1993). The MAP kinase cascade is essential for diverse signal transduction pathways. *TIBS* **17**: 128-30.

substrate-specific. Listed in Table 3.1 are the substrates for which IphP activity was measured in both the absence and presence of heparin. Heparin was inhibitory toward most substrates examined, but it enhanced IphP activity toward casein (^{32}P-Ser) and MAPK (^{32}P-Thr / ^{32}P-Tyr) by as much as 10-fold. This effect was dose-dependent (Fig. 3.2). The enhancement was most readily apparent with heparin; however, other polyanions could mimic the effect to various degrees (Table 3.3).

Heparin lowers the apparent K_M of IphP toward MAPK (^{32}P-Thr / ^{32}P-Tyr)

Kinetic studies revealed that heparin enhances IphP activity toward MAPK (^{32}P-Thr / ^{32}P-Tyr) by lowering the apparent K_M about 4-fold while having little effect on k_{cat} (Table 3.2). Kinetic parameters could not be obtained for casein (^{32}P-Ser) because the K_M was apparently too high for accurate determination,. This likely reflects the low stoichiometry to which the cAMP-dependent protein kinase phosphorylates casein (5).

MAPK (^{32}P-Thr /^{32}P-Tyr), casein (^{32}P-Ser) and IphP associate with heparin

Since heparin increases IphP activity toward MAPK (^{32}P-Thr /^{32} P-Tyr), and presumably casein (^{32}P-Ser), by increasing the apparent affinity of the enzyme for these substrates, we asked whether heparin physically interacts with either IphP, these substrates, or both. A striking correlation was found between the ability of a substrate to bind heparin-agarose and the effect of heparin on the dephosphorylation of the substrate by IphP. IphP, MAPK (^{32}P-Thr / ^{32}P-Tyr), casein (^{32}P-Ser), and angiotensin II (^{32}P-Tyr) were each found to bind heparin-agarose, while casein (^{32}P-Tyr) and several other non-enhancable substrates did not (Table 3.4). These results suggest that heparin lowers the apparent K_M of IphP toward MAPK (^{32}P-Thr / ^{32}P-Tyr) and casein (^{32}P-Ser) by recruiting the enzyme and substrate into the same microenvironment, effectively increasing their concentrations. Conversely, IphP activity is reduced toward proteins and peptides which do not bind heparin due to the effective removal of IphP from solution by the polyanion, resulting in an apparent decrease in [IphP]. The fact that angiotensin II (^{32}P-Tyr) binds to the heparin-agarose column and yet is inhibited by heparin may be explained by the relatively small size of this peptide: both IphP and angiotensin II (^{32}P-Tyr) are recruited to the same microenvironment by heparin, but angiotensin II (^{32}P-Tyr) likely is too small to be readily accessible to the active site of IphP while bound to the polyanion. The associative model is presented diagramatically in Figure 3.3.

The association between heparin and IphP, casein, or MAPK was detected using sucrose density gradient ultracentrifugation (Fig. 3.4). Proteins peaks readily visible in the absence of heparin were shifted in the presence of the polyanion. The dispersion of the

[5]McGowan, C. H., and P. Cohen. (1988). *Methods Enzymol*. **159**: 416-26.

protein peaks for IphP and MAPK over a broad area suggests that these proteins associate with heparin molecules of widely varying sizes, whereas the explicit shift of the casein peak suggests a preference by this protein for heparin molecules of a particular size range.

Proteins with high pI values block enhancement by heparin

The associative model described above was tested by a "competition" experiment. We reasoned that if heparin enhances IphP activity toward MAPK (^{32}P-Thr / ^{32}P-Tyr) and casein (^{32}P-Ser) by recruiting the proteins to the same microenvironment, then it should be possible to obstruct enhancement by removing available heparin. This was accomplished by adding proteins of various pI values to the reaction mixture in 10-fold excess to heparin. As predicted by the model, proteins expected to bind heparin during the reaction, i.e., proteins with a net positive charge at pH 5.0, hindered heparin-induced enhancement of IphP activity toward MAPK (^{32}P-Thr / ^{32} P-Tyr) and casein (^{32}P-Ser), while those proteins bearing a negative charge at pH 5.0 did not (Figure 3.5).

Membrane lipids do not mimic heparin enhancement

The first 24 amino acids of IphP constitute a signal sequence that targets the protein for secretion into the periplasmic or extracellular space *in vivo*. Indeed, the enzyme is recovered in the growth media when expressed in *E. coli* (6). We reasoned, therefore, that heparin, a macromolecular polyanion, may mimic naturally- occuring constituents of the membrane or sheath of cyanobacteria. Thus, we examined the effect of various membrane lipids and cyanobacterial sheath components on IphP activity *in vitro*. As shown in Table 3.5, no membrane or sheath components tested displayed a dramatic effect on IphP activity toward casein (^{32}P-Ser); however, phosphotidylglycerol and phosphatic acid enhanced IphP activity 2.0 - 2.5-fold.

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

Table 3.1. Listed are proteins and peptides which were tested as potential substrates for IphP and the specific enzyme activity measured for each. Assays were performed in 25µL of 50mM sodium acetate, pH 5.0, containing 2mM DTT and 10-800ng of IphP. All substrates were tested at 2µM protein-bound [^{32}P]phosphate. The effect of heparin, 0.1mg/mL, on enzyme activity was determined for these substrates that were dephosphorylated by IphP. [a]nd, not detectable (less than 10pmol/min/mg).

Table 3.1
Relative Activity of IphP toward Protein/Peptide Substrates

Substrate	Enzyme Activity (pmol/min/mg)	Effect of Heparin (% activity without heparin)
casein (^{32}P-Ser)	40	910
casein (^{32}P-Tyr)	7000	10
RCML (^{32}P-Ser)	5050	29
RCML (^{32}P-Tyr)	245	13
poly (Glu_4Tyr) (^{32}P-Tyr)	117	39
MAPK (^{32}P-Thr / ^{32}P-Tyr)	3667	650
MBP (^{32}P-Tyr)	3450	1
MBP (^{32}P-Thr / ^{32}P-Ser)	2083	9
R_{II} (^{32}P-Ser)	1250	5
histone H2a (^{32}P-Ser)	nd[a]	-
histone H2b (^{32}P-Ser)	nd	-
YINAS (^{32}P-Tyr)	520	13
angiotensin-II (^{32}P-Tyr)	111	76
kemptide (^{32}P-Ser)	nd	-

Table 3.2. Listed are the kinetic parameters of IphP toward selected substrates. All assays were performed under standard conditions (see Materials and Methods) employing 10-800ng IphP. MAPK (^{32}P-Thr / ^{32}P-Tyr) was assayed in both the absence and presence of 0.1mg/mL heparin.

Table 3.2
Kinetic Parameters of IphP toward Selected Substrates

Substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}M^{-1}$)x10^{-6}
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 (^{32}P-Thr / ^{32}P-Tyr)	0.32	400	1.25
MAP kinase (^{32}P-Thr / ^{32}P-Tyr)(+heparin)	0.081	300	3.75
RCML (^{32}P-Tyr)	0.009	10.5	1.12
RCML (^{32}P-Ser)	0.041	167	4.07
YINAS (^{32}P-Tyr)	0.050	24	0.48

Table 3.3. Other polyanions also enhance IphP activity toward casein (P-Ser) and MAPK (P-Thr / P-Tyr), but less dramatically than heparin. Each polyanion was tested at a final concentration of 0.1 mg/mL. a, 100% = 40pmol/min/mg; b, 100% = 3600pmol/min/mg.

Table 3.3
The Effect of Various Polyanions on IphP Activity

	Relative Activity (%)	
Effector	**Casein (P-Ser)**	**MAPK (P-Thr / P-Tyr)**
none	100[a]	100[b]
heparin	860	604
chondroitin sulfate	150	275
poly (Asp)	200	110
ssDNA	250	200

Table 3.4. IphP, casein (^{32}P-Ser), MAPK (^{32}P-Thr/^{32}P-Tyr), and angiotensin II (^{32}P-Ser) were each retained on a column of heparin-agarose under standard phosphatase assay conditions. Approximately 0.1-0.5mg of each protein listed was applied to a 1mL column of heparin-agarose equilibrated with 50mM sodium acetate, pH 5.0, containing 2mM DTT. Proteins were eluted and detected as described under Materials and Methods. Eluted pNPP was detected by the addition of IphP (~500ng) to fractions and observing the formation of a yellow color (see Materials and Methods).

Table 3.4
Affinity of IphP and Substrates toward Heparin-agarose

Protein	Bind to Heparin-agarose Column
IphP	YES
casein (^{32}P-Ser)	YES
casein (^{32}P-Tyr)	NO
MAPK (^{32}P-Thr / ^{32}P-Tyr)	YES
RCML (^{32}P-Ser)	NO
RCML (^{32}P-Tyr)	NO
MBP	NO
poly (Glu,Tyr)(^{32}P-Tyr)	NO
YINAS (^{32}P-Tyr)	NO
angiotensin II (^{32}P-Tyr)	YES
pNPP	NO

Table 3.5. The effect of membrane lipids and cyanobacterial sheath components on IphP activity toward casein (^{32}P-Ser) was examined. IphP (~800ng) was assayed under standard reaction conditions (see Materials and Methods) except that each of the above components were added at a final concentration of 1 mg/mL. Lipid micelles were formed by sonication. a, 100%=40pmol/min/mg.

Table 3.5
Effect of Membrane & Sheath Components on IphP Activity

Lipid / Sheath Component	Relative Activity (%)
None	100[a]
phosphotidylglycerol	260
phosphatic acid	200
cardiolipin	156
phosphotidylserine	149
phosphotidylcholine	110
phosphotidylinositol	90
UTEX sheath material	100
DRH1 sheath material	100

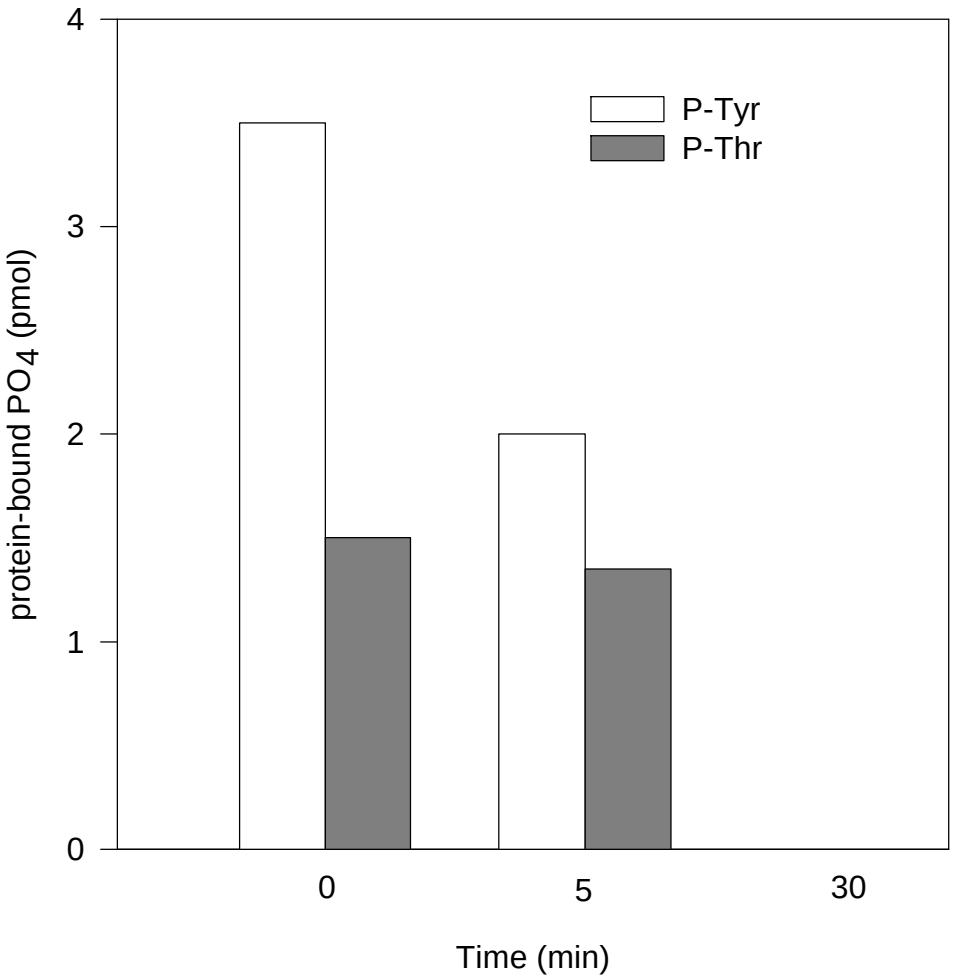

Figure 3.1

IphP dephosphorylates P-Tyr from MAPK (P-Thr / P-Tyr) faster than P-Thr. Shown are the quantities of phosphothreonine and phosphotyrosine recovered upon phosphoamino acid analysis of [^{32}P] MAPK (P-Thr / P-Tyr) following incubation with IphP (~50ng) under standard reaction conditions (see Materials and Methods) for the times indicated.

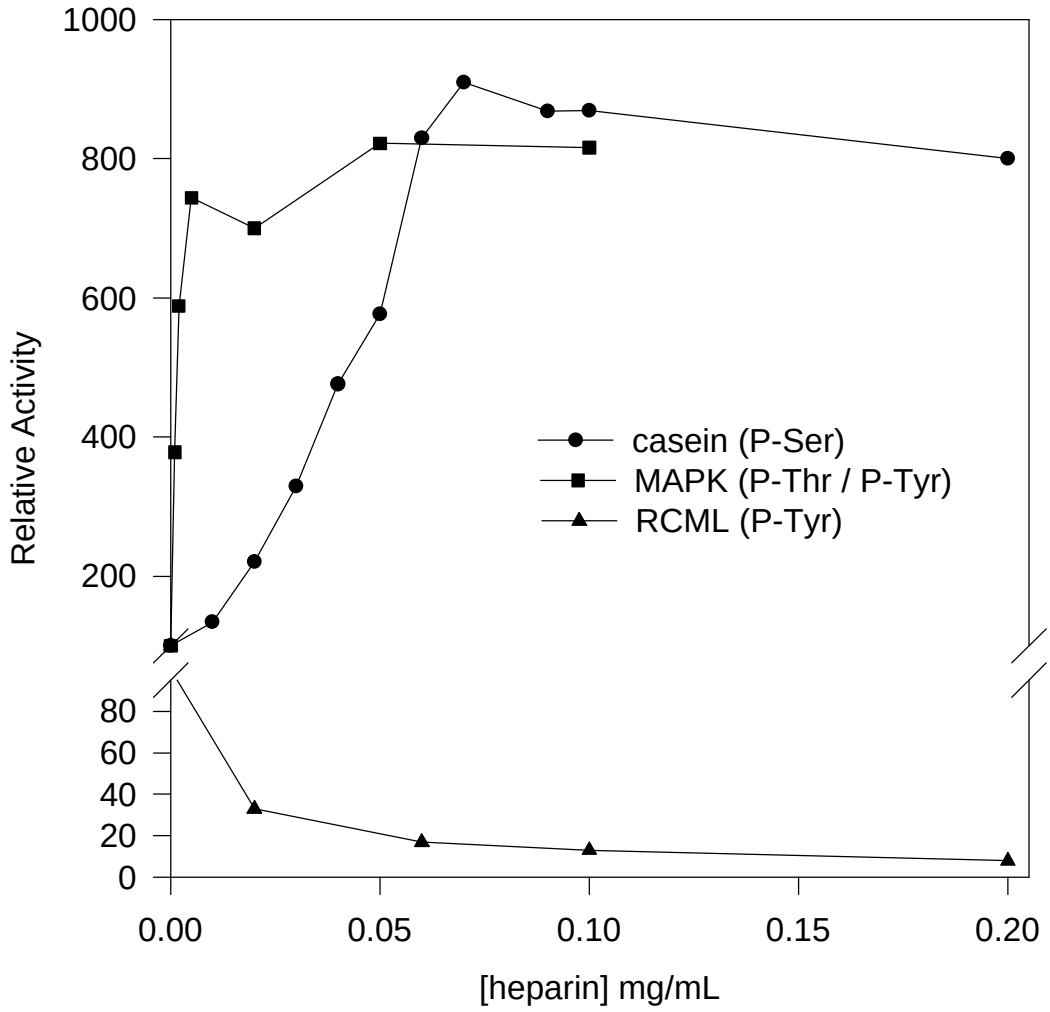

Figure 3.2

Enhancement of IphP activity toward casein (^{32}P-Ser) and MAPK (^{32}P-Thr / ^{32}P-Tyr) by heparin is concentration-dependent. Shown is the activity of IphP (10-800 ng) toward casein (^{32}P-Ser), MAPK (^{32}P-Thr / ^{32}P-Tyr), and RCML (^{32}P-Tyr) in the presence of the indicated concentrations of heparin. Activity in the absence of heparin (i.e., [heparin]=0mg/mL) is set to 100% (~40pmol/min/mg for casein (^{32}P-Ser), ~3600pmol/min/mg for MAPK (^{32}P-Thr/ ^{32}P-Tyr), and ~250pmol/min/mg for RCML (^{32}P-Tyr)).

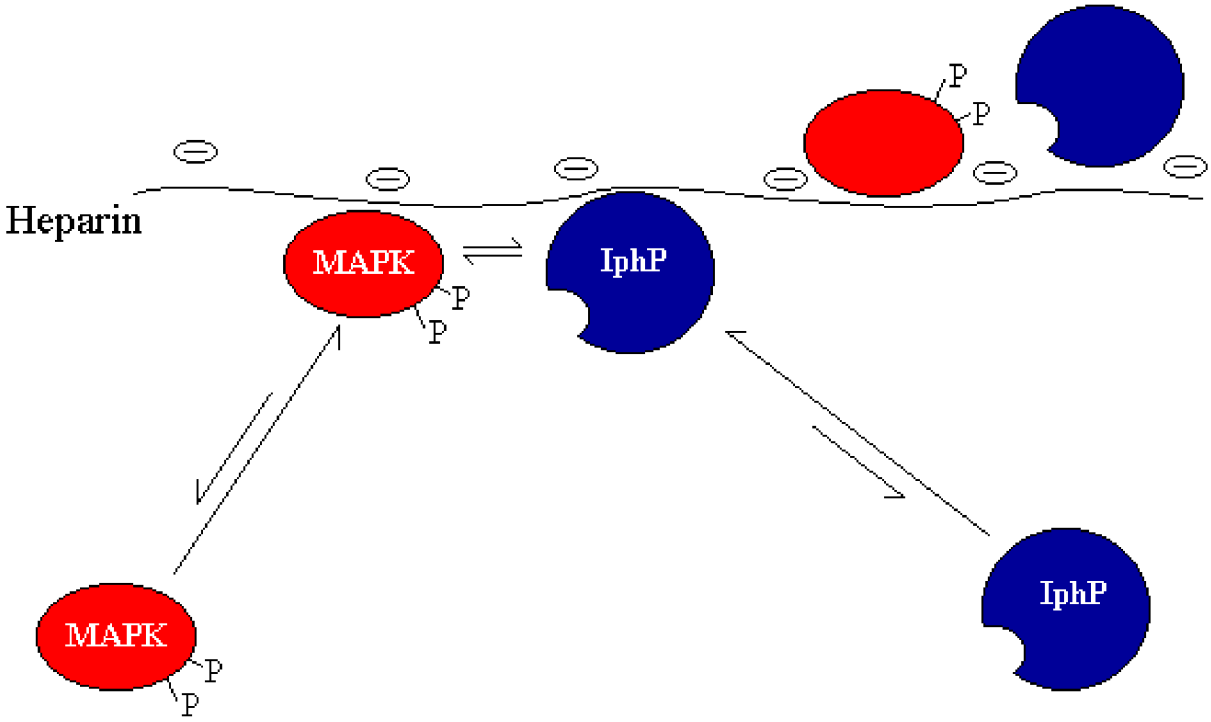

Figure 3.3

Associative model for the enhancement of IphP activity toward MAPK (P-Thr / P-Tyr) by heparin. Red = MAPK (P-Thr / P-Tyr); blue = IphP. Both enzyme and substrate associate with the polyanion.

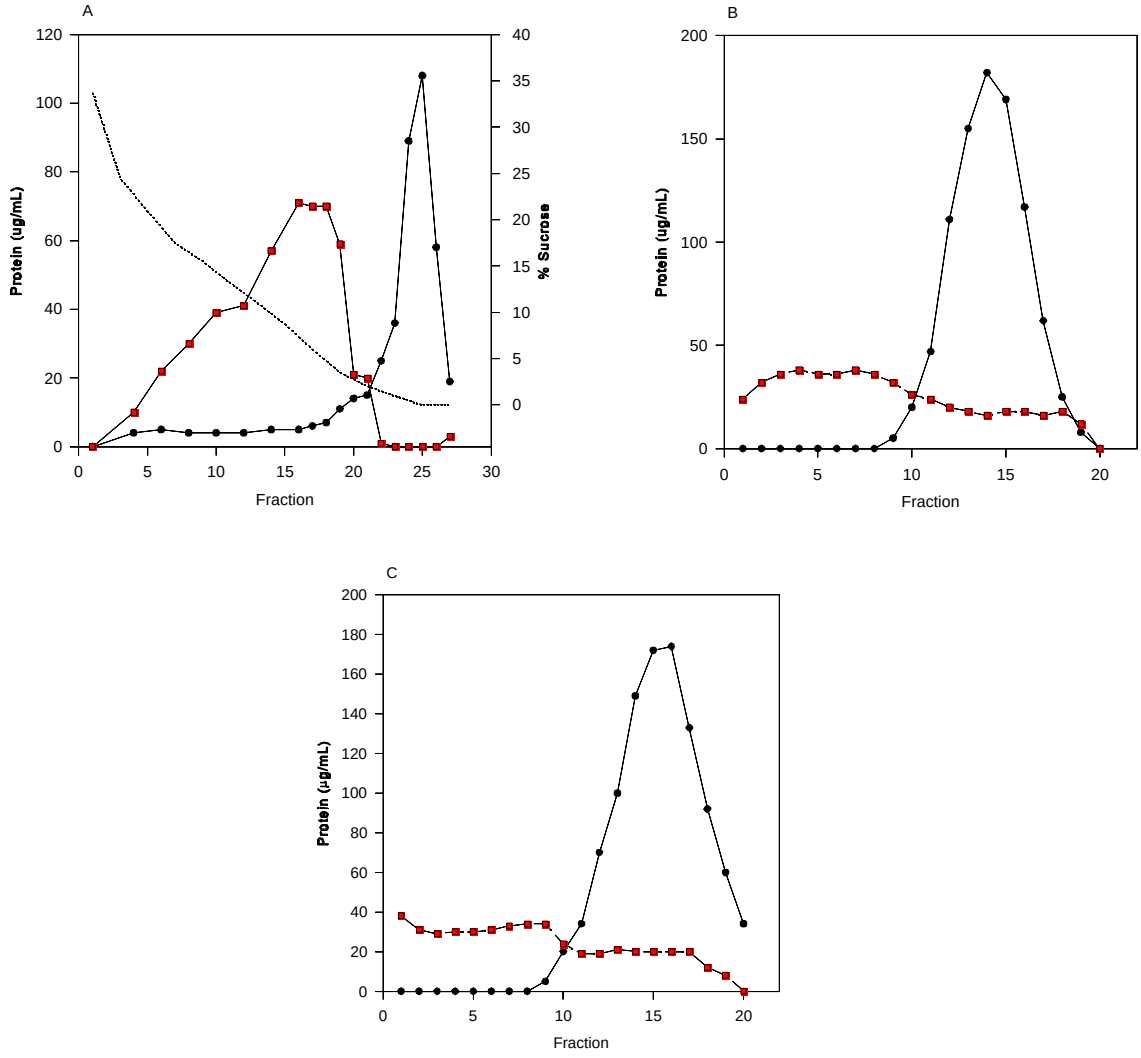

Figure 3.4

Sucrose density gradient ultracentrifugation of heparin-protein complexes. Proteins (~1mg) with or without 1mg heparin were applied to the top of a sucrose gradient prepared as described under Materials and Methods. Ultracentrifugation was conducted overnight as described under Material and Methods. A, migration of casein (●) and casein-heparin complex (■) through the sucrose gradient. B, migration of MAPK (●) and MAPK-heparin complex (■) through gradient. C, migration of IphP (●) and IphP-heparin complex (■) through the gradient.

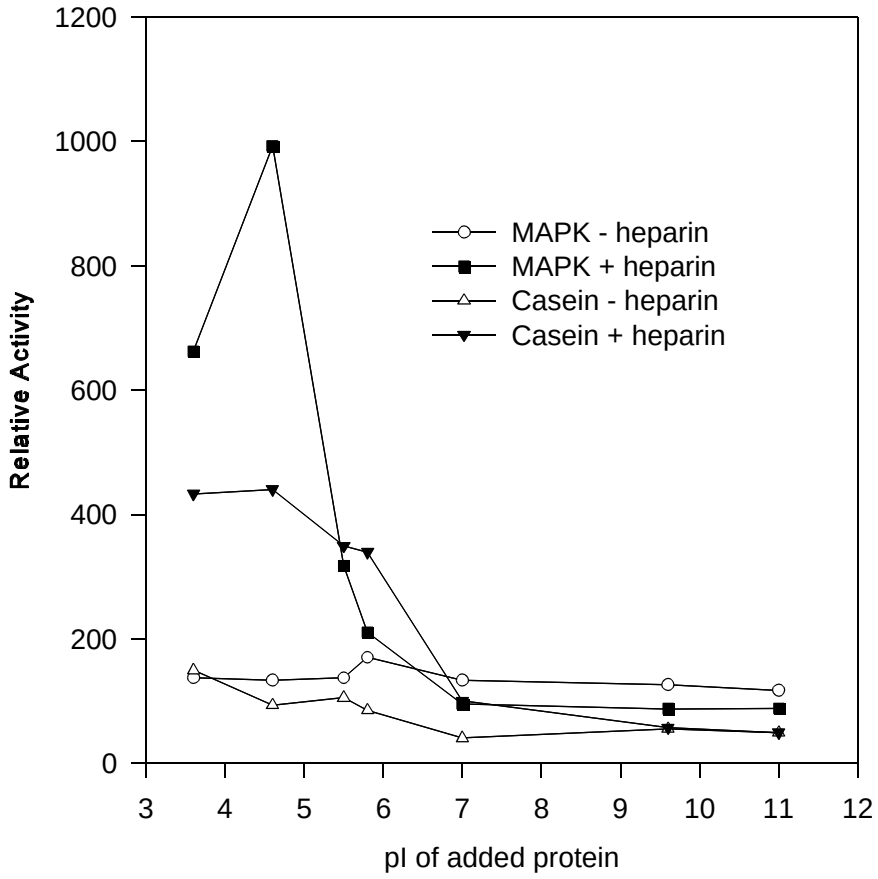

Figure 3.5

Proteins with high pI values block heparin enhancement. Shown are the relative rates of dephosphorylation of casein (^{32}P-Ser) and MAPK (^{32}P-Thr/^{32}P-Tyr) by IphP (10-800 ng) in the absence and presence of 0.1mg/mL heparin with or without the following proteins (1mg/mL final concentration): amyloglucosidase (pI 3.6), ovalbumin (pI 4.6), BSA (pI 5.5), catalase (pI 5.8), hemoglobin (pI 7.0), cytochrome c (pI 9.6), or lysozyme (pI 11.0).