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City University of New York PH.D. 1984

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PHOSPHOTYROSYL-PROTEIN PHOSPHATASES

by

JONATHAN CHERNOFF

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1984

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Abstract

PHOSPHOTYROSYL-PROTEIN PHOSPHATASES

by

Jonathan Chernoff

Adviser: Professor Heng-Chun Li

The phosphorylation of proteins at tyrosine has been implicated in the establishment and maintenance of neoplastic transformation by certain retroviruses, as well as in the regulation of normal cell growth by various polypeptide hormones. Although overall phosphorylation levels are determined by a balance of protein kinase and phosphatase activities, much more attention has been given to the study of tyrosyl-protein kinases than to the corresponding phosphotyrosyl-protein phosphatases. Therefore, a survey was undertaken to determine what enzymatic species possess phosphotyrosyl-protein phosphatase activity. Purified enzymes, including human prostatic acid phosphatase (an enzyme which has traditionally been assayed using the phosphotyrosine analog p-nitrophenyl phosphate) and bovine heart phosphoprotein phosphatases-1-4, were examined for phosphotyrosyl-protein phosphatase activity. In addition, extracts of bovine heart and other mammalian tissues were examined for detection of specific phosphotyrosyl-protein phosphatases. Two such enzymes were

purified and characterized.

The results of this study indicate that: (a) acid phosphatases selectively dephosphorylate phosphotyrosyl-proteins; (b) phosphoprotein phosphatases-2, -3, and -4, but not -1, display activity toward phosphotyrosyl-proteins, but this activity is much less than that toward phosphoseryl- or phosphothreonyl-proteins; (c) mammalian tissues contain several phosphatase isozymes which are relatively specific for phosphotyrosyl-proteins. These include three species separable by anion-exchange chromatography, termed phosphotyrosyl-protein phosphatase Y-1, -2, and -3. When measured at neutral pH in the presence of EDTA, these species represent 33, 55, and 12%, respectively, of the total phosphotyrosyl-protein phosphatase activity in bovine heart extract. Phosphatase Y-1 (M_r 13,000), does not bind to DEAE-cellulose at pH 7.0, has an acidic pH optimum, and is associated with acid phosphatase activity. Phosphatase Y-2 (M_r 65,000) elutes from DEAE-cellulose at 0.1-0.2 M KCl, has a neutral pH optimum, is stimulated by chelating agents, and has little activity toward p-nitrophenyl phosphate.

In conclusion, (a) all enzymes active toward p-nitrophenyl phosphate appear also to be active toward phosphotyrosyl-proteins; (b) the classical phosphoprotein phosphatases-1-4 have relatively little phosphotyrosyl-protein phosphatase activity; and (c)

extracts of mammalian tissues contain multiple species of specific phosphotyrosyl-protein phosphatases.

ACKNOWLEDGEMENTS

I dedicate this thesis to my father, who would have been proud to read it.

I would like to thank Dr Terry Krulwich for her unfailing support and my advisor, Dr Heng-Chun Li, for giving me the freedom to make my own mistakes.

FORWARD

Portions of this thesis have been published in the following papers:

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Chernoff J Li H-C Cheng Y-SE Chen LB (1983) Characterization of a Phosphotyrosyl Protein Phosphatase Activity Associated with a Phosphoseryl Protein Phosphatase of M_r=95,000 from Bovine Heart. J Biol Chem 258:7852-7857

Chernoff J Li H-C (1983) Multiple Forms of Phosphotyrosyl- and Phosphoseryl-Protein Phosphatase from Cardiac Muscle: Partial Purification and Characterization of an EDTA-Stimulated Phosphotyrosyl-Protein Phosphatase. Arch Biochem Biophys 226:517-530

Li H-C Chernoff J Chen LB Kirschonbaum A (1984) A Phosphotyrosyl-Protein Phosphatase Activity Associated with Acid Phosphatase from Human Prostate Gland. Eur J Biochem 138:45-51

Chernoff J Sells MA Li H-C (1984) Characterization of Phosphotyrosyl-Protein Phosphatase Activity Associated with Calcineurin. Biochem Biophys Res Comm in press

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Li H-C Chernoff J Kirschonbaum A Chen LB (1983) Prostatic Acid Phosphatase is a Major Phosphotyrosyl-Protein Phosphatase Activity in Human Prostate Gland. Fed Proc 42:2028

Chernoff J Li H-C (1984) Ca^{2+}, Calmodulin and Mg^{2+}-Dependent Dephosphorylation of Phosphotyrosyl-Proteins by Calcineurin. Fed Proc 43:766

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ABBREVIATIONS

Tyr-protein, Tyrosyl-protein; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; ts, temperature-sensitive; RSV, Rous sarcoma virus; (P)Tyr, phosphotyrosyl; (P)Ser, phosphoseryl; (P)Thr, phosphothreonyl; PNPP, p-nitrophenyl phosphate; NP40, Nonidet P-40; MES, 4-morpholinoethanesulfonic acid; TCA, trichloroacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; DTT, dithiothreitol; BSA, bovine serum albumin; EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; SDS, sodium dodecyl sulfate.

INTRODUCTION

The reversible phosphorylation of proteins is an important and well-established mechanism of metabolic regulation in eukaryotic organisms (for review, see Ref. 1). The most common acceptor sites in protein for the incorporation of phosphate are serine and threonine (2). Until 1979, these were the only stable phosphoamino acids detected in protein. In that year, a novel class of protein kinase was described (3), which specifically phosphorylates proteins at tyrosine, and it has become clear that this class of kinase is likely to play a central role in the initiation and maintenance of transformation by certain retroviruses (4). Tyrosyl protein (Tyr-protein) kinase activity has also been found to be associated with the receptors for various growth-promoting hormones, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and insulin, and thus may participate in the control of normal cellular growth and development (5-8).

If tyrosine phosphorylation is truly part of a regulatory mechanism for cell growth, enzymes should exist which can reverse this modification. The first experimental evidence for such an activity was obtained by measuring total

phosphotyrosine levels in cells infected with temperature-sensitive (ts) mutants of Rous sarcoma virus (RSV). In such cells, the phosphotyrosine levels were shown to return to normal within 1-2 h of shifting to the nonpermissive temperature (9). This decrease could not be attributed to protein turnover and thus indicated the presence of endogenous phosphotyrosyl ((P)Tyr)-protein phosphatase activity.

It is clear from examples of other systems that employ protein phosphorylation as a means of regulation that both the protein kinases and phosphatases are usually under tight control. In general, alterations in levels of phosphorylation result from simultaneous changes in protein kinase and phosphatase activities (10). Therefore, it is likely that the level of tyrosine phosphorylation will be mediated by a complex interplay between both Tyr-protein kinases and (P)Tyr-protein phosphatases. While the Tyr-protein kinases have been intensively studied in recent years and something is known of their structure and regulation, less attention has been paid to the corresponding (P)Tyr-protein phosphatases. The isolation and characterization of these (P)Tyr-protein phosphatases is the subject of this thesis.

1.1 Tyrosyl-Protein Kinases

1.1.1 Onc Gene Products

A number of retroviruses which are able to induce neoplastic transformation of appropriate tissue culture cells have been shown to contain nucleotide sequences unnecessary for viral replication but essential for the induction and maintenance of the transformed state (for review, see Ref. 11). These RNA sequences have been termed v-onc genes and are believed to have arisen from normal cellular sequences (c-onc genes) by recombination between viral and cellular information (11). These cellular sequences are well-conserved in evolution and so are presumed to serve an important function in normal cells. The molecular biology of the onc genes has been the subject of intense scrutiny in recent years, but the mechanism by which the products of the v-onc genes initiate the process of transformation remains obscure.

1.1.1.1 $pp60^{src}$

$pp60^{src}$, [1] the protein encoded by the transforming gene of (RSV) was the first onc gene product to be described and remains the most thoroughly studied of these proteins. This

---

1. unless otherwise indicated, $pp60^{src}$ is used to refer to the protein product of the v-src gene

protein was identified in 1977 by Erikson and coworkers (12,13) as an M_r 60,000 virally encoded antigen in RSV-transformed cells and as an *in vitro* translation product of viral mRNA. The following year this group demonstrated that incubation of the *src* gene product with anti-pp60^{src} antibodies in the presence of [γ-^{32}P]ATP resulted in phosphorylation of the M_r 52,000 IgG heavy chain, suggesting that pp60^{src} might be associated with protein kinase activity (14). In addition, the purified protein itself became phosphorylated upon incubation with [γ-^{32}P]ATP, indicating possible autophosphorylation: a characteristic common to many protein kinases (15). Later studies showed that (a) kinase activity copurified with pp60^{src} (16-18); (b) RSV mutants *ts* in the *src* gene contained thermolabile kinase activity (14,19-21); and (c) *Escherichia coli* containing plasmids bearing the *src* gene produced functional pp60^{src} (22,23). Thus, several lines of evidence indicate that the *src* gene indeed codes for a protein kinase.

In 1980, Hunter and Sefton demonstrated the the target amino acid in proteins phosphorylated by pp60^{src} was tyrosine; not threonine as originally reported (24). The failure to detect this phosphoamino acid previously was due to the paucity of this substance in normal cells (0.03% of total protein-bound phosphate in chick embryo fibroblasts) and the comigration of phosphotyrosine with phosphothreonine

in the electrophoretic separation procedures then employed (9). This group further showed that cells infected with RSV contained about 10-fold higher levels of phosphotyrosine associated with protein than uninfected cells and that this finding was not a general characteristic of cells transformed by other agents (9). In cells infected with ts mutants of RSV, the level of tyrosine phosphorylation rose 10-fold upon shifting the temperature from nonpermissive to permissive values and returned to normal under the converse condition (9). These and other related findings gave rise to the hypothesis that the phosphorylation of proteins at tyrosine plays a primary role in the malignant transformation of cells by RSV. This model envisions that pp60^{src}, by phosphorylating certain host proteins, initiates a cascade of reactions which culminate in the the manifold changes characteristic of malignant cells. Since normal cells contain a functional analog of this protein (pp60$^{c\text{-}src}$), the events triggered by pp60^{src} are thought to be due to either quantitative or qualitative differences between the viral and host enzymes (for review, see Ref. 25).

In addition to pp60^{src}, Tyr-protein kinase activity has been associated with the protein products of several other onc genes, including abl, fes, yes, fps, and ros (26-30) (Table 1). Portions of the protein products of these genes

Table 1 Viral *onc* genes and their protein products

Gene	Probable species of origin	Protein kinase	Tyrosyl protein kinase	plasma membrane associated
src	chicken	+	+	+
fps	chicken	+	+	+
yes	chicken	+	+	+
ros	chicken	+	+	+
abl	mouse	+	+	+
fes	cat	+	+	+
fms	cat	+	+	-
erbB	chicken	-	-	+
raf	mouse	-	-	?
sis	monkey	-	-	?
myb	chicken	?	?	?
myc	chicken	-	-	-
rel	turkey	?	?	?
mos	mouse	-	-	-
ras	rat	+	-	+
bas	mouse	?	?	?
fos	mouse	-	-	?

bear strong homology (31-36) to one another, particularly in the C-terminal region, which contains the protein kinase domain (31-36). This family of Tyr-protein kinases is of ancient lineage and is distantly related to cAMP-dependent protein kinase (37). Three other onc genes, erb-B, mos, and raf, also encode protein structurally related to pp60^{src} (38-40), but these products have yet to be shown to contain Tyr-protein kinase activity. Interestingly, the predicted sequence of the erb-B gene has recently been shown to bear close resemblance to the Tyr-protein kinase domain of the EGF receptor (see section 1.1.3). Whether the failure to detect kinase activity in these gene products is due to inadequate assay techniques or to genuine lack of enzymatic activity is unknown.

1.1.2 Polypeptide Hormone Receptors

The mechanisms by which the binding of polypeptide hormones to specific receptors induce the physiological effects of these hormones have long been sought. Recently, evidence has accumulated implicating Tyr-protein kinase activity as a possible component of this process for EGF, PDGF, and insulin.

1.1.2.1 Epidermal Growth Factor Receptor

In 1979, Cohen *et al* reported that certain proteins in the plasma membrane of A-431 cells underwent phosphorylation following treatment of these membranes with EGF (41). The increase in phosphorylation activity was shown not to be due to effects on ATPase activity nor to growth factor inhibition of dephosphorylation reactions (41). Subsequently, this group showed that the EGF receptor itself was among the proteins phosphorylated and that both the EGF-stimulated kinase and receptor copurified on EGF affinity chromatography (42). The phosphorylation of the receptor was shown to occur at tyrosine (43). These findings indicated that Tyr-protein kinase activity might play a role in normal cell growth and were consistent with the idea that the virally encoded Tyr-protein kinases, which induce neoplastic growth, might represent an aberrant variation of a normal regulatory process.

1.1.2.2 Platelet-Derived Growth Factor Receptor

PDGF is a potent mitogen for a variety of cultured cells of mesenchymal and glial origin (44). The PDGF receptor from human fibroblasts appears to be a membrane protein of $M_r \simeq$ 185,000 (45). Like the EGF receptor, the PDGF receptor has been shown to possess intrinsic Tyr-protein kinase activity (45), and is autophosphorylated at tyrosine *in vivo*. As

will be discussed (section 1.1.5), the PDGF and EGF receptor/kinase share certain endogenous protein substrates and may therefore act through similar pathways.

1.1.2.3 Insulin Receptor

The insulin receptor is a tetrameric glycoprotein consisting of two M_r 135,000 (α) and two M_r 95,000 (β) subunits in a disulfide-linked complex (46-49). In 1982, Kasuga et al reported that a very early event following insulin binding to its receptor is enhanced phosphorylation of the insulin receptor itself (50). This phosphorylation occurs on the β-subunit, whereas insulin binding takes place on the α-subunit. In isolated receptors, tyrosine is the site of phosphorylation (51). The receptor preparation also displays Tyr-protein kinase activity toward exogenous substrates (52-54). The kinase and receptor activities were found to be inseparable throughout a variety of isolation techniques and it is generally accepted that the β-subunit of the insulin receptor, like the EGF and PDGF receptors, possesses intrinsic Tyr-protein kinase activity (55-59). Although the effect of autophosphorylation of the receptor on kinase activity is imperfectly understood, it is possible that this modification augments kinase activity toward exogenous substrates. Rosen et al demonstrated that incubation of partially purified insulin receptor with ATP,

Mg^{2+}, and insulin coincided with the generation of an activated, insulin-independent, receptor/kinase, and that the time required for activation was consistent with that needed for insulin-dependent self-phosphorylation of the receptor (60).

Evidence suggesting that the receptor/kinase activity may be fundamental to insulin's mode of action include the finding that substances known to mimic insulin action in intact cells also share insulin's ability to stimulate the kinase activity of purified receptors and that an antagonist of insulin action blocks this phosphorylation (61). In addition, Tamura et al reported that treatment of partially purified rat adipocyte insulin receptor with trypsin, at concentrations previously shown to activate glycogen synthase, resulted in an increase in the tyrosine phosphorylation of exogenous substrates and in receptor-derived peptides (62). This group also showed that vanadate, a known inhibitor of (P)Tyr-protein phosphatase activity, enhanced the degree of phosphorylation of the insulin receptor as well as activated glycogen synthase in isolated rat adipocytes (63). Although not conclusive, these findings taken together imply a link between insulin receptor/kinase activity and the intracellular effects of this hormone.

1.1.3 Relationship Between Growth Factors, Growth Factor Receptors, and Onc Gene Products

The predicted amino acid sequence of p28^{sis}, the transforming gene product of simian sarcoma virus, has been found to have homology to that of PDGF (64,65). Presumably, the sis gene product is able to bind to the PDGF receptor and stimulate Tyr-protein kinase activity. This finding raises the intriguing possibility that the simian sarcoma virus, though encoding an onc gene product unrelated to Tyr-protein kinases, nevertheless induces neoplastic transformation by exploiting metabolic pathways similar or identical to those used by retroviruses which directly encode Tyr-protein kinases. A related, but somewhat different, discovery of this kind has recently come to light concerning the erb-B gene. Portions of the EGF receptor have been sequenced, revealing significant homology to the predicted sequence of the erb-B gene product (66). Interestingly, the erb-B gene encodes a protein homologous to the transmembrane and protein kinase domains of the EGF receptor, but not the EGF-binding domain. These findings suggest that the erb-B gene represents a truncated version of the EGF receptor gene, and that the transforming potential of its gene product may be related to unregulated tyrosine phosphorylation. Although kinase activity has not been directly associated with the erb-B gene product, it is

interesting to note that cells transformed by avian erythroblastosis virus contain elevated levels of tyrosine phosphorylation (4). Thus, more than half of the known onc genes encode Tyr-protein kinases, growth factors whose effects upon binding to specific receptors include activation of receptor Tyr-protein kinase activity, or homologues to the receptors for these growth factors. It therefore seems likely that the phosphorylation of substrates at tyrosine is intimately related to the transformation processes initiated by these onc genes. It will be of much interest to compare the sequences of additional growth factors and their receptors with various onc gene products whose function at present remains unknown.

1.1.4 Tyrosyl-Protein Kinase Substrates

Unravelling the connection between Tyr-protein kinase activity and the regulation of cellular growth and development will depend on the identification of the endogenous substrates for tyrosine phosphorylation. One method which has been used to identify endogenous (P)Tyr-proteins is one- and two-dimensional gel electrophoresis of $^{32}P_i$-labelled extracts of RSV-infected cells, followed by base-treatment of the gel to hydrolyze most (P)Ser/Thr-proteins (67-69). The most prominent of the

^{32}P-Tyr-proteins resolved by this technique is a protein of M_r 36,000 (70-73). Determining the function of this protein has proven a more formidable task than its identification, and thus far among the few clues are that (a) the M_r 36,000 protein is found in many, but not all tissues (74), (b) this protein is also phosphorylated at tyrosine in cells infected with a variety of other oncogenic retroviruses, as well as in EGF treated A-431 cells (75-79), and (c) this protein is associated with the plasma membrane (80-82). Rubsamen et al proposed that the M_r 36,000 protein is identical to cytosolic malic dehydrogenase (83), but other laboratories have been unable to confirm this finding (80,84-86). Recently, an M_r 85,000 protein from membrane vesicles of porcine intestinal epithelial cells has been shown to be composed of two M_r 36,000 and one M_r 10,000 subunits (87). The M_r 36,000 subunits are identical to the protein of this molecular weight which is phosphorylated in RSV-transformed cells. The M_r 85,000 protein binds in a Ca^2-dependent manner to F-actin and non-erythroid spectrin, suggesting that the phosphorylation of this polypeptide may affect the actin-spectrin network. Other prominent ^{32}P-Tyr-proteins resolved by two-dimensional gel electrophoresis include species of M_r 46,000, 42,000, and 28,000 (68,69). The M_r 46,000 and 28,000 proteins were subsequently identified as the glycolytic enzymes enolase and phosphoglycerate mutase

(see below) while the M_r 42,000 species has not been associated with a known enzyme. This phosphoprotein has been found both in chick cells transformed by retroviruses which encode Tyr-protein kinases and in normal chick cells treated with diverse mitogenic agents, including EGF, PDGF, serum, trypsin, and the tumor promoters 12-O-tetradecanoyl-phorbol-13-acetate and teleocidin (68,75,88-90). Thus, the tyrosine phosphorylation of M_r 42,000 protein could be important in the regulation of cell division, although direct evidence for this supposition is lacking.

Vinculin, an M_r 130,000 cytoskeletal protein which is thought to anchor actin microfilaments to the cell membrane (92,93), has been identified as an endogenous substrate for pp60$^{\underline{src}}$ (94). Only about 1% of vinculin molecules are so modified in RSV-transformed fibroblasts, and it is not known whether this small amount of phosphorylation accounts for the large alterations in cytoskeletal structure characteristic of transformed cells (for review, see Ref. 95). Furthermore, not all transforming retroviruses which encode Tyr-protein kinases cause an increase in vinculin phosphorylation *in vivo* (96,97).

Other proteins which become phosphorylated at tyrosine upon transformation by RSV include enolase, phosphoglycerate

mutase and lactate dehydrogenase (84). As with vinculin, only a small percentage of rhese molecules are modified, and as yet no changes in enzymatic activity accompanying tyrosine phosphoryation have been reported.

1.2 Phosphatases

Enzymes exhibiting phosphatase activities historically have been divided into two categories: those whose activities are measured using small, nonprotein, phosphoesters, such as p-nitrophenyl phosphate (PNPP) and those which are measured using phosphoprotein substrates. Enzymes active toward the former substrates often exhibit pH optima at acidic or alkaline values, and are collectively termed acid and alkaline phosphatases, respectively, while enzymes active toward the latter are termed phosphoprotein phosphatases. The two classes of phosphatase are not mutually exclusive. Some phosphoprotein phosphatases, as will be discussed, are able to dephosphorylate nonprotein substrates, with maximal activity at non-neutral pH, while certain so-called acid and alkaline phosphatases are able to act upon phosphoprotein substrates. Therefore, the classification of phosphatases into these two broad categories is not meant to imply absolute substrate specificity, and is retained here for convenience.

1.2.1 Acid and Alkaline Phosphatases

Acid and alkaline phosphatases have been studied since the the early part of this century and much is known of their structures and catalytic properties. Although measurements of acid and alkaline phosphatase levels are clinically useful in the evaluation of many human diseases, the physiologic role of these enzymes has never been determined. A vast body of literature exists concerning these enzymes (for reviews, see Refs. 98,99) of which a short summary is provided in the following paragraphs.

1.2.1.1 Acid Phosphatases

Acid phosphatases are ubiquitous in distribution and multiple isozymes of this enzyme have been described in mammalian tissues (99). These isozymes can be grouped into two categories: high molecular weight ($M_r \simeq 100,000$), dimeric molecules, such as the human prostatic enzyme, and low molecular weight ($M_r \simeq 14,000$), monomeric, enzymes, such as those from bovine or human liver or erythrocytes (99-105). In addition to size differences, the high and low molecular weight acid phosphatases differ from one another in several respects. The low molecular weight species are soluble cytosolic enzymes whereas the larger acid phosphatases are probably lysosomal (106). The former are insensitive to inhibition by L(+)-tartrate and NaF;

compounds which are potent inhibitors of most of the high molecular weight enzymes (107,108). The low molecular weight enzymes are markedly sensitive to reagents which interact with sulhydryl groups, such as p-chloromercurobenzoic acid, and heavy metal ions, such as Hg^{2+} and Ag^{2+} (99,102,107-110), while the larger enzymes are much less sensitive to these compounds. Finally, the low molecular weight enzymes are believed to have essential active site cysteine residues, in contrast to the high molecular weight enzymes, which have histidine at the active site (111).

Of the high molecular weight acid phosphatases, the human prostatic acid phosphatase (M_r 100,000) has been the subject of particular interest since its assocation with prostatic carcinoma more than 40 years ago (112). The levels of this enzyme in prostate are extremely high (0.86 mg/g of wet tissue) (113) and therefore it is presumed that this enzyme serves a vital, though as yet undescribed, function in this tissue. Although the endogenous substrates for this enzyme are not known, prostatic acid phosphatase is active *in vitro* toward a variety of phosphoesters as well as certain (P)Ser/Thr-proteins (99,114,115).

Human erythrocytes contain an $M_r \simeq 14,000$ acid phosphatase which is known to be encoded by a gene on chromosome 2 (116,117). An enzyme of this molecular weight was later

identified in many other tissues which displayed similar catalytic properties and electrophoretic mobility as the erythrocyte enzyme (118,119). It was claimed, therefore, that the erythrocyte enzyme has widespread tissue distribution (118,119). However, unlike the low molecular weight acid phosphatases from bovine liver and brain, the erythrocyte acid phosphatase is inhibited by formaldehyde and is active toward β-glyceryl phosphate (99,120,121). Thus, at present, the relationship between the low molecular weight acid phosphatases from human erythrocyte and from other tissues such as liver and brain is unclear.

1.2.1.2 Alkaline Phosphatases

Mammalian alkaline phosphatases exist in several isozymic forms, with molecular weights ranging from 60,000 to 200,000 (98). In man, these enzymes are the protein products of three different genes, encoding the placental, intestinal, and bone, kidney, and liver isozymes, respectively (122). All these enzymes are membrane-bound glycoproteins, exist in dimeric form comprised of two very similar or identical subunits, have an optimum of a pH around 11 to 12, do not require a reducing reagent for activity and are inhibited by organ-specific inhibitors such as L-phenylalanine and L-homoarginine (98). These enzymes are structurally and catalytically distinct from the PNP phosphatase activity

associated with protein phosphatases-2, -3, and -4 (see section 1.2.2).

The function of alkaline phosphatase probably differs from tissue to tissue. In bone, this activity is thought to be concerned with ossification, while in other tissues, alkaline phosphatase may have a direct role in the transport of nutrients across the epithelial membrane (98). Recently, it has been suggested that these enzymes may act physiologically as (P)Tyr-protein phosphatases (see section 1.2.4.1).

1.2.2 Classical Protein Phosphatases [1]

The cytosolic protein phosphatases found in extracts of bovine cardiac muscle and other tissues may be divided into four types, as summarized in Table 2 (123). This classification is based upon substrate specificity, sensitivity to heat-stable inhibitor proteins, and response to various regulatory molecules. Although there is some disagreement among different laboratories concerning the identity of various molecular forms of protein phosphatases in a given tissue, activities similar to the bovine heart phosphatases-1, -2, -3, and -4 described in the following

---

1. The term 'protein phosphatase' will be used in place of the more formally correct 'phosphoprotein phosphatase' for the sake of brevity

Classical Protein Phosphatases

Protein Phosphatase	M_r	Activators	PNPP Activity
-1	61 k	ATP/Mg + F_A	trace
-2	80 k	Ca/calmodulin	high
-3A	156 k	Mn > Mg	high
B	161 k		
C	95 k		
-4	49 k	Mg	medium

sections have been found in extracts of muscle and liver by other laboratories and have been termed protein phosphatases-1, -2B, -2A, and -2C, respectively, by Cohen and his colleagues (for a review, see Ref. 10).

1.2.2.1 Protein Phosphatase-1

Phosphatase-1 (M_r 61,000) dephosphorylates the b-subunit of phosphorylase kinase faster than the a-subunit and is sensitive to inhibition by heat-stable inhibitor-1 (in its phosphorylated form) and inhibitor-2 (124-128). Phosphatase-1 can be activated by ATP-Mg^{2+} and an activator protein (M_r 45,000) termed F_A, which has been shown to be identical to glycogen synthase kinase-3 (129-132). Phosphatase-1 is composed of a 1:1 complex of an M_r 38,000 catalytic subunit and inhibitor-2 (133). The activation of this enzyme is envisioned to occur as a result of phosphorylation (and subsequent dissociation) of inhibitor-2 by F_A in the presence of ATP-Mg^{2+} (134). Phosphatase-1 can also be partially activated by Mn^{2+} or Co^{2+} alone, but not by Mg^{2+} (135). Phosphatase-1 is thought to represent the major phosphorylase phosphatase in most tissues (136). The native enzyme and its catalytic subunit possess PNP phosphatase activity [2], but this activity is much less than

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2. H. -C. Li, unpublished observations

that associated with protein phosphatases-2, -3, and -4 (see following sections).

1.2.2.2 Protein Phosphatase-2

Phosphatase-2 (calcineurin) is an M_r 80,000 Ca^{2+}/calmodulin stimulated enzyme which is found in high concentrations in brain (137). This enzyme consists of an A (M_r 61,000) and B (M_r 19,000) subunit (138). The latter of these two subunits binds Ca^{2+} and is believed to exert a regulatory influence on the phosphatase activity, while the A subunit probably contains the catalytic domain (139,140). Phosphatase-2 displays little activity toward phosphorylase a, but readily dephosphorylates inhibitor-1 in vitro, accounting for about 70% of the total potential activity on inhibitor-1 in skeletal muscle extracts (136). It has been proposed that, in contracting muscle, phosphatase-2 (activated by Ca^{2+} influx) serves to dephosphorylate inhibitor-1, thereby activating phosphatase-1. In brain, phosphatase-2 is found principally in the caudate nucleus and putamen, in association with post-synaptic densities and the microtubules of dendrites (141). Phosphatase-2 levels have been shown to increase during synaptogenesis (142). In light of these findings, and because glycogen metabolism has a relatively minor role in the brain, it is likely that the function of phosphatase-2 differs in skeletal and neural

tissues. Phosphatase-2 has recently been shown to possess activity toward PNPP and other small phosphoesters (143,144), but the functional significance of this activity is unknown.

1.2.2.3 Protein Phosphatase-3

Phosphatase-3A, -3B, and -3C (M_r 156,000, 161,000, and 95,000) each contain an identical M_r 35,000 catalytic subunit, in combination with various other subunits (145-147). Treatment of the native molecules with 6 M urea or 80% ethanol at room temperature results in the dissociation of the catalytic subunit, concomitant with an increase in enzymatic activity (145,148). Phosphatase-3 isozymes have broad substrate specificity and contain an intrinsic PNP phosphatase activity. Interestingly, this PNP phosphatase activity differs in several respects from the protein phosphatase activity. These differences include the following: the PNP phosphatase activity has an alkaline pH optimum, requires Mg^{2+} and a reducing reagent such as 2-mercaptoethanol, and is relatively heat-labile, whereas the phosphorylase phosphatase activity has a neutral pH optimum, does not require additional cofactors and is relatively heat-stable (149,150). Nevertheless, these two activities co-purify and have identical mobility on polyacrylamide gel electrophoresis. In addition, when the

high molecular weight native phosphatase-3 isozymes are treated with ethanol or urea, the two activities are both transformed to an M_r 35,000 form (147).

1.2.2.4 Protein Phosphatase-4

Phosphatase-4 is a Mg^{2+}-dependent enzyme, consisting of a single M_r 49,000 subunit (151,152). This enzyme was originally reported to be relatively specific for glycogen synthase, but further study has shown that phosphatase-4 has a broad substrate specificity (10). In liver, phosphatase-4 accounts for the majority of activity toward phosphorylated HMGCoA reductase and pyruvate kinase. This phosphatase may therefore play a role in various central metabolic pathways. Like phosphatase-2 and -3, this enzyme has readily detectable PNP phosphatase activity. [3]

1.2.3 Phosphothreonyl-Protein Phosphatases

Relatively few reports have appeared concerning phosphatases specific for (P)Thr-proteins. Pinna et al described a rat liver cytosolic "casein phosphatase" which preferentially hydrolyzed phosphothreonine residues in casein which contained both phosphoserine and phosphothreonine residues (154). This group later partially

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3. J. Chernoff. and H. -C. Li, unpublished observations

purified this enzyme (renamed phosphatase-T) and showed that it catalyzed the dephosphorylation of a synthetic peptide containing ^{32}P-threonine, but not the identical peptide in which this residue was replaced by ^{32}P-serine (155). By contrast, protein phosphatase-1 was active toward both phosphopeptides. Phosphatase-T was shown to be inactive toward other ^{32}P-Ser-peptides as well as ^{32}P-Tyr-IgG, indicating that its preference for phosphothreonyl residues was independent of substrate. These findings, as well as the observed response of this enzyme to various inhibitors, indicate that phosphatase-T is a specific (P)Thr-protein phosphatase, distinct from other previously described phosphatases.

1.2.4 Phosphotyrosyl-Protein Phosphatases

1.2.4.1 Activity of Acid/Alkaline Phosphatases toward Phosphotyrosyl-Proteins

In 1981, Swarup et al demonstrated that alkaline phosphatases from several sources selectively dephosphorylated (P)Tyr-proteins (156). This group later showed that the particulate fraction from TCRC-2 cells (a subclone of HeLa cells) contained a (P)Tyr-protein phosphatase activity which co-eluted with alkaline phosphatase activity from wheat-germ lectin-Sepharose and histone-Sepharose columns (157). These activities were

inhibited by vanadate. Because alkaline phosphatases are associated with the plasma membrane, a site where many of the Tyr-protein kinases and their substrates are located, and because the pH optimum for this enzyme's activity toward (P)Tyr-proteins is shifted toward neutral values, these enzymes have been postulated to have an important role in metabolic circuits involving tyrosine phosphorylation.

In 1982, Leis and Kaplan reported that an acid phosphatase in the plasma membrane of human astrocytoma showed marked specificity toward ^{32}P-Tyr-histone and that this activity could be inhibited by vanadate (158). Li et al subsequently reported similar findings using human prostatic acid phosphatase (Ref. 159 and section 4.1.1). These findings raise the possibility that all phosphatases active toward PNPP will also dephosphorylate (P)Tyr-proteins. The structural similarity between PNPP and the phosphotyrosine residue support this hypothesis.

1.2.4.2 Activity of Classical Protein Phosphatases toward Phosphotyrosyl-Proteins

Preliminary experiments in this laboratory indicated that partially purified bovine heart protein phosphatases-3C (M_r 95,000) and -4 (M_r 49,000)) are able to catalyze the dephosphorylation of ^{32}P-Tyr-IgG (160). These enzymes, however, were much more active toward (P)Ser/Thr-proteins

than (P)Tyr-proteins. Foulkes et al also found that protein phosphatase-3A and -3C as well as their M_r 35,000 catalytic subunits were active toward ^{32}P-Tyr-casein, but that, taken together, this activity represented only about 10% of the total activity in chicken brain extract (161).

The ability of protein phosphatases-1-4 to dephosphorylate (P)Tyr-proteins was studied in detail by the author, and will be presented in the Results and Discussion sections.

1.2.4.3 Phosphotyrosyl-Protein Phosphatase Activity in Tissue Extracts

Using membrane vesicles from A-431 cells, Brautigan et al reported that the M_r 150,000 EGF receptor underwent rapid dephosphorylation upon incubation at 30°C and that this dephosphorylation could be almost completely prevented by the addition of 10 μM Zn^{2+} (162). Other divalent cations were ineffective. Similarly, membrane vesicles from normal and RSV-transformed rat cells were shown to contain Zn^{2+}-inhibited, (P)Tyr-protein phosphatase activity (163). The effect of Zn^{2+} was reversible, and inhibition of dephosphorylation was observed with a variety of endogenous vesicle (P)Tyr-proteins, suggesting that this divalent cation interacted with the phosphatase rather than its substrates, although a specific interaction between Zn^{2+} and phosphotyrosine residues in protein could not be ruled out.

Similar findings were reported by Foulkes and coworkers (164) who showed that crude extracts of rat muscle and liver and contained ^{32}P-Tyr-IgG phosphatase activity which was sensitive to inhibition by Zn^{2+}, but not by F^- or inhibitor-2. These properties were in marked contrast to the ^{32}P-Ser-phosphorylase phosphatase activity in these extracts.

The author has characterized a number of (P)Tyr-protein phosphatases from bovine heart and other tissues (Results 4.3.3) which may be related to (P)Tyr-protein phosphatases described by other laboratories. A detailed review of the purification and properties of (P)Tyr-protein phosphatases from a variety of tissues and a comparison of these activities to those described by other laboratories will be presented in the Discussion section of this thesis. A brief outline is provided in the following paragraphs.

In 1982, Horlein et al partially purified a Zn^{2+}-sensitive, (P)Tyr-protein phosphatase from Ehrlich Ascites Tumor cells (165). The enzyme was purified by using DEAE-Sephadex, Zn^{2+}-affinity, and Sephadex G-75 chromatography. The partially purified enzyme was inhibited by μM concentrations of Zn^{2+}, but not by EDTA, F^-, or tetramisole, a specific inhibitor of alkaline phosphatases (166). The enzyme had an M_r of 40,000 and a pH optimum at

6.5-7.0, and was active toward a variety of (P)Tyr-proteins but not (P)Ser-phosphorylase or PNPP. The enzyme became markedly unstable following the DEAE-Sephadex purification step.

Using ^{32}P-Tyr-casein as substrate, Foulkes *et al* examined extracts of chicken brain and found three major peaks of (P)Tyr-protein phosphatase activity on DEAE-cellulose chromatography (161). These enzymes were termed T-1, T-2, and T-3. These enzymes were shown to be distinct from the (P)Ser-protein phosphatases present in this tissue. Maximal activity was observed in the presence of EDTA while divalent cations such as Mg^{2+} and Mn^{2+} were inhibitory. The major peak of activity (T-2) eluted from DEAE-cellulose at about 145 mM NaCl and by gel filtration had a molecular weight of about 43,000. Phosphatases T-1, T-2, and T-3 may be related to three (P)Tyr-protein phosphatases found in bovine heart (Ref. 123 and Results 4.3). [4]. The relationship of these EDTA-stimulated species to one another and to the Zn^{2+}-inhibited (P)Tyr-protein phosphatase described by Horlein *et al* is not known.

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4. These bovine heart enzymes have been termed Y-1, Y-2, and Y-3 in order to conform to the established abbreviation for tyrosine and to avoid confusion with the (P)Thr-protein phosphatase-T described by Pinna and associates (155)

1.2.4.5 Phosphotyrosine Phosphatases

Phosphotyrosine exists in high levels in Drosophila and is thought to represent a storage form for tyrosine (167). Fukami and Lipmann (168) have described an enzyme from Drosophila which dephosphorylates free L-phosphotyrosine. This enzyme has a high molecular weight ($M_r \simeq 170,000$) and is strongly inhibited by F^-. No attempt was made to determine whether this enzyme is also active toward (P)Tyr-proteins. Interestingly, this enzyme was shown to catalyze the reversible transfer of phosphate from phosphotyrosine to tyrosine. In this regard, the Drosophila phosphotyrosine phosphatase resembles a phosphoserine phosphatase described previously (169,170).

SPECIFIC AIMS

Because the enzymes responsible for dephosphorylating (P)Tyr-proteins were almost entirely unknown at the outset of this project, the author wished to address the following basic questions:

1. What activity do PNP phosphatases, such as the well-studied human prostatic acid phosphatase, have towards (P)Tyr-proteins? How specific is this activity? Is the (P)Tyr-protein phosphatase activity attributable to the prostatic enzyme, or to a contaminating phosphatase?

2. What activity, if any, do protein phosphatases-1-4 from bovine heart have toward (P)Tyr-proteins? What are the catalytic requirements for this activity, and do these requirements resemble those for dephosphorylating PNPP? How specific is this activity?

3. Do specific (P)Tyr-protein phosphatases exist in bovine heart, distinct from the aforementioned enzymes? If so, what is the subcellular distribution of these enzymes, and are these enzymes unique to the tissue examined? What are the biophysical and biochemical characteristics of these enzymes?

EXPERIMENTAL PROCEDURES

3.1 Materials

[γ-^{32}P]ATP was purchased from Amersham. Phosphoserine, -threonine, -tyrosine, α-casein, and myosin light chain were from Sigma. Phosphorylase b (171), glycogen synthase (172), and heat-stable inhibitors-1 and -2 (173,174) were purified from rabbit skeletal muscle. Histone H2b was from Worthington.

3.2 Preparation of Protein Kinases

Rabbit skeletal muscle phosphorylase kinase was purchased from Sigma. The catalytic subunit of cAMP-dependent protein kinase was purified from bovine heart (157). Plasma membranes enriched in EGF receptor/kinase were prepared from A-431 cells (41,162). The preparation is free of detectable Ser(Thr)-protein kinase activity.

Antisera specific for pp60$^{v\text{-}src}$ were prepared by inducing tumors in newborn rabbits with RSV, Schmidt-Ruppin strain, group D (12). An immunoaffinity resin specific for pp60$^{v\text{-}src}$ was prepared by partially purifying IgG from the antisera by 50% $(NH_4)_2SO_4$ precipitation, followed by coupling to CNBr-activated Sepharose 4B (Pharmacia) (18). The pp60$^{v\text{-}src}$ was partially purified from a RSV-transformed cell line,

AnAn (67), by the following procedures. AnAn cells were suspended in a buffer containing 10 mM KP_i, pH 7.0, 40% glycerol, 0.02% Nonidet P-40 (NP40), 2 mM EDTA, 1 mM EGTA, 100 mM KCl, 2 mM 2-mercaptoethanol and 1% aprotinin. The cells were disrupted in a Dounce homogenizer and the suspension was centrifuged for 30 min at 30,000 x g. The supernatant was filtered through glass wool and applied to an aminohexyl agarose (Sigma) column equilibrated in Buffer A (5 mM KP_i, pH 7.0, 20% glycerol, 0.05% NP40, 1 mM EDTA, and 1 mM 2-mercaptoethanol). The column was washed with 5 volumes of Buffer A plus 0.4 M KCl and the pp60$^{\underline{v\text{-}src}}$ was eluted by 2 column volumes of Buffer A plus 1 M KCl. The enzyme solution was extensively dialyzed against Buffer A and applied to a DEAE-Sephacel (Pharmacia) column equilibrated with the same buffer. The column was extensively washed with Buffer A and the pp60$^{\underline{v\text{-}src}}$ was eluted by 2 column volumes of Buffer A plus 0.2 M KCl. The enzyme solution was immediately applied to an immunoaffinity column. The column was washed with 5 volumes of Buffer A plus 0.2 M KCl and 1.5 M KSCN. The enzyme was immediately dialyzed against Buffer A for 2 h followed by Buffer A plus 30% glycerol and stored at -20°C. The pp60$^{\underline{v\text{-}src}}$ so obtained is free from protein kinase activity which phosphorylates serine or threonine residues.

3.3 Preparation of Phosphatases

3.3.1 Isolation of Human Prostatic Acid Phosphatase

Acid phosphatase was purified from prostate glands obtained from patients with benign hypertrophy. The purification scheme was designed on the basis of the method of Lam *et al* (114). Prostatic tissue was stored at -70°C. About 2 g of frozen tissue was homogenized in 10 ml of distilled water. The homogenate was subjected to 4 freeze-thaw processes followed by centrifugation at 100,000 x *g* for one hour. The supernatant was dialyzed against 500 ml of Buffer B (20 mM Tris-HCl, pH 7.4, 10 mM 2-mercaptoethanol, 1 mM EDTA) overnight. The dialyzed supernatant was chromatographed on a DEAE-cellulose (Whatman DE-52) column (1.6 x 20 cm) equilibrated with Buffer B. The column was developed with 100 ml Buffer A followed by 200 ml of a linear NaCl gradient from 0.05 to 0.5 M. A minor peak of (P)Tyr-protein phosphatase activity was detected in the Buffer B wash. A major active peak, which represented 90% of the enzymatic activity recovered from the resin, was eluted between 0.22 - 0.25 M NaCl. In both peaks, the (P)Tyr-protein phosphatase precisely coincided with the acid phosphatase activity. The active fractions of the major peak were pooled for further purification. The enzyme solution was adjusted to pH 4.0 by adding 0.1 M citric acid

and solid ammonium sulfate was added to the supernatant to 55% saturation. The precipitate formed was removed by centrifugation. Ammonium sulfate was then added to the supernatant to 75% saturation. The precipitate was collected, dissolved and dialyzed against 10 mM Tris-HCl, pH 8.0. Finally, the enzyme solution was applied to a DEAE-cellulose column (1 x 10 cm) equilibrated with 10 mM Tris-HCl, pH 8.0. The column was developed with 200 ml of a linear NaCl gradient from 0 to 0.25 M NaCl. A single peak containing both phosphatase activities, which was eluted between 0.1 and 0.13 M NaCl, was pooled, concentrated by membrane ultrafiltration (Amicon, PM 10) and stored at $-20^{o}C$.

3.3.2 Isolation of Protein Phosphatases-1-4

Protein phosphatase-1 (F_C) (176) and its protein activator (F_A) (131), as well as protein phosphatases-3 (150) phosphatase-2 (calcineurin) and calmodulin were purified from bovine brain (177).

3.3.3 Isolation of (P)Tyr-Protein Phosphatases Y-1 and Y-2

Bovine heart was obtained from a local slaughter house and stored at $-20^{o}C$. Frozen tissue was thawed, freed of fat and connective tissue, cut into small pieces and homogenized for 30 s at low speed in a Waring blender with 2.5 vol of

extraction buffer (20 mM Tris-HCl, pH 7.0, 2 mM EGTA, 0.1 mM phenylmethylsulfonyl flouride). The homogenate was centrifuged at 15,000 x g for 20 min and the supernatant was filtered through glass wool. Solid ammonium sulfate was added to the extract to 55% saturation. The precipitated protein was collected by centrifugation, suspended in a small volume of Buffer C (20 mM Tris-HCl, pH 7.0, 10 mM 2-mercaptoethanol, 0.1 mM EDTA) plus 50 mM KCl and dialyzed against the same buffer. For separation of the phosphatase isozymes, the 55% $(NH_4)_2SO_4$ fraction derived from about 200 g of tissue was loaded on a DEAE-cellulose (DE-52, Whatman) column (2.5 x 40 cm) presaturated with Buffer C plus 50 mM KCl. After sample application the column was rinsed with 1 bed volume of the same buffer and developed with 1.5 liters of a linear salt gradient from 0.05 to 0.4 M KCl in Buffer C. Twelve milliliter fractions were collected. Aliquots were withdrawn for the analysis of phosphatase activities (Fig. 15).

3.3.3.1 Phosphatase Y-1

For the purification of (P)Tyr-protein phosphatase Y-1 from bovine heart, the DEAE-cellulose column loaded with the 55% $(NH_4)_2SO_4$ fraction was washed with Buffer B plus 0.05 M KCl until the absorbance of the eluate at 280 nm became less than 0.01. The eluate was concentrated by precipitation with

70% $(NH_4)_2SO_4$, dialyzed against Buffer D (20 mM Tris-maleate, pH 6.5, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 1 mM KP_i, 0.05 M KCl) plus 20% glycerol, and applied to a Sephadex G-75 column (2.5 x 90 cm) equilibrated in the same buffer. The major active peak (Fractions 55-70, Fig. 16a) was pooled and applied to a SP-sephadex (Pharmacia) column (2.5 x 20 cm) presaturated with buffer D plus 20% glycerol. The column was washed with 1 bed volume of Buffer D plus 20% glycerol and eluted with 900 ml of a linear gradient of KCl from 0.05 to 1.0 M. The active fractions (74-84, Fig. 16b) were pooled and dialyzed against buffer D. The pooled activity was then applied to a Matrex Red A (Amicon) column (1.3 x 20 cm) equilibrated with Buffer D. The column was developed with 200 ml of 0 to 20% glycerol gradient. The active fractions (23-34, Fig. 16c) were pooled, concentrated by membrane ultrafiltration (PM 10, Amicon), dialyzed against Buffer B plus 50% glycerol, and stored at -20°C.

3.3.3.2 Phosphatase Y-2

For the purification of (P)Tyr-protein phosphatase Y-2 from bovine heart, the DEAE-cellulose column loaded with the 55% $(NH_4)_2SO_4$ fraction was eluted with Buffer C plus 0.05 M KCl until the absorbance of the eluate at 280 nm became less than 0.01 (Fig. 18a). The (P)Tyr-protein phosphatase activity was then eluted from the column with Buffer C plus

0.12 M KCl, precipitated with 70% $(NH_4)_2SO_4$, dialyzed against Buffer C plus 0.05 M KCl, and rechromatographed on a second DEAE-cellulose column. The column was eluted with a linear salt gradient from 0.05 to 0.2 M KCl in Buffer C (Fig. 18b). The major active peak was pooled, concentrated with 70% $(NH_4)_2SO_4$, and dialyzed against Buffer E (20 mM Tris-HCl, pH 7.0, 10 mM 2-mercaptoethanol, 0.1 M KCl, 20% glycerol) and loaded onto a Sephacryl S-200 column (2.6 x 50 cm) for elution with the same buffer. The active peak (Fractions 52-63, Fig. 18c) was pooled and applied onto a poly-L-lysine-Sepharose (Sigma) column (1.5 x 25 cm) presaturated with Buffer E. The column was eluted with 1 bed volume of Buffer E followed by 500 ml of a linear gradient of KCl from 0.1 to 0.5 M. The major (P)Tyr-protein phosphatase peak (Fractions 31-38, Fig. 18d) was pooled, concentrated by membrane ultrafiltration (PM 10, Amicon), dialyzed against 20 mM Tris-HCl, pH 7.0, 10 mM 2-mercaptoethanol, 50 mM KCl, 50% glycerol, and stored at $-20^{\circ}C$. The enzyme preparation obtained from the poly-L-lysine-Sepharose step was used in all studies of (P)Tyr-protein phosphatase Y-2 unless otherwise specified.

3.3.4 Other Phosphatases

Human placental alkaline phosphatase and human acid phosphatases, bands 2 (seminal fluid), 3 and 4 (leukocyte),

5a and 5b (serum), were from Calbiochem. Wheat germ acid phosphatase and bovine kidney alkaline phosphatase were from Worthington.

3.4 Preparation of ^{32}P-Labeled Proteins

3.4.1 High Specific-Activity, Low Concentration Substrates

(A) ^{32}P-Tyr-IgG: The pp60$^{\underline{v\text{-}src}}$ in AnAn cells was immunocomplexed with antisera and collected on protein A-Sepharose beads (Pharmacia) (67). Phosphorylation of the heavy chain of IgG was carried out by incubating the protein A-Sepharose beads (1.5 ml wet volume) with 20 mM Tris-HCl, pH 7.2, 5 mM $MgCl_2$, and 1 mCi of [γ-^{32}P]ATP (6660 cpm/fmol) at 20°C for 20 min. The protein A-Sepharose beads were then washed repeatedly with RIPA buffer (20 mM Tris-HCl, pH 7.2, 0.15 M NaCl, 1% Triton X-100, 0.1% SDS, and 1% deoxycholate) to remove unbound radioactivity. The ^{32}P-labeled complexes were separated from protein A-Sepharose by extracting with a small volume of 0.005 N HCl and dialyzed against 20 mM Tris-HCl, pH 7.2.

(B) ^{32}P-Tyr-casein: Phosphorylation of α-casein with the partially purified pp60$^{\underline{v\text{-}src}}$ was carried out at 20°C for 60 min in an incubation mixture containing 0.1 M MES, pH 6.5, 1 mM $MnCl_2$, 0.4 μM [γ-^{32}P]ATP (6660 cpm/fmol), 1 mg/ml α-casein and 70 μg/ml pp60$^{\underline{v\text{-}src}}$. The reaction was

terminated by the addition of an equal volume of 25% TCA. The precipitated ^{32}P-Tyr-casein was extensively washed with 25% TCA and suspended in a small volume of distilled H_2O. The suspension was then adjusted to pH 7.0 with 1 N NaOH followed by extensive dialysis against 20 mM Tris-HCl, pH 7.2.

(C) ^{32}P-Thr-inhibitor-1 and ^{32}P-Ser-casein: The phosphorylation reactions were carried out at 30°C for 30 min in an incubation mixture containing 50 mM Tris-HCl, pH 7.0, 5 mM $MgCl_2$, 0.5 unit/ml of the catalytic subunit of cAMP-dependent protein kinase, 0.4 μM [γ-^{32}P]ATP (6660 cpm/fmol), 0.5 mg/ml of inhibitor-1 or 1 mg/ml of α-casein. The reactions were terminated and the ^{32}P-proteins were washed as described in the preceding paragraph.

(D) ^{32}P-Ser-phosphorylase a: 1 mg/ml crystalline phosphorylase b was incubated with 50 mM Tris-HCl, pH 8.6, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 1.2 units/ml of phosphorylase kinase, and 0.4 μM [γ-^{32}P]ATP (6660 cpm/fmol). After incubating at 30°C for 30 min, 5 volumes of cold, saturated $(NH_4)_2SO_4$ solution, pH 7.0, were added to the reaction mixture. ^{32}P-Ser-phosphorylase a was collected by centrifugation at 4°C, dissolved in a small volume of 20 mM Tris-HCl, pH 7.0, 10 mM 2-mercaptoethanol, and reprecipitated by 5 volumes of the $(NH_4)_2SO_4$ solution. This

washing was repeated 5 times and the ^{32}P-Ser-phosphorylase *a* obtained was dialyzed against 20 mM Tris-HCl, pH 7.0, 10 mM 2-mercaptoethanol and stored at 4°C.

(E) A-431 membrane proteins: Phosphorylated A-431 membrane proteins were prepared according to Swarup *et al* (156).

When these phosphoproteins were used as substrates, one unit of phosphatase activity was defined as the amount of enzyme catalyzing the release of 1 fmol $^{32}P_i$/min at 30°C.

3.4.2 Low Specific-Activity, High Concentration Substrates

(A) ^{32}P-Tyr-casein, ^{32}P-Tyr-histone, and ^{32}P-Tyr-myosin light chain: 2 mg/ml -casein, histone H2b, or myosin light chain was incubated with 0.15 mg/ml A-431 membrane, 2 µg/ml EGF, 0.3 mM [γ-^{32}P]ATP (3050 cpm/pmol), 2 mM $MnCl_2$, 0.1 mM ammonium vanadate, 0.2% NP40 and 20 mM Hepes, pH 7.4, in a volume of 0.3 ml for 6 hrs at 30°C. The reaction was terminated by the addition of an equal volume of 25% TCA and the precipitated protein was washed as described in the preceding paragraphs

(B) ^{32}P-Ser-casein and ^{32}P-Thr-inhibitor-1: α-casein and inhibitor-1 were incubated with [γ-^{32}P]ATP and cAMP-dependent protein kinase as previously described (178).

(C) ^{32}P-Ser-phosphorylase *a*: phosphorylase *b* was

phosphorylated by phosphorylase kinase as reported previously (179).

When these phosphoproteins were used as substrates, one unit of phosphatase activity was defined as the amount of enzyme catalyzing the release of 1 nmol $^{32}P_i$/min at 30°C.

Phosphoamino acid analysis (see section 3.7) indicated that the $^{32}P_i$ was incorporated into the specified amino acid residue in each ^{32}P-protein prepared.

3.5 Phosphatase Assay

Phosphatase activity was assayed at 30°C by determining the release of $^{32}P_i$ from ^{32}P-protein (180). The standard assay (25 µl) contained 50 mM Na-acetate (pH 5.0) or Tris-HCl (pH 7.4 or 8.6), 0.5 mM dithiothreiotol (DTT), 2 mg/ml bovine serum albumin (BSA), and the indicated concentrations of ^{32}P-protein. Enzyme concentrations and assay times were adjusted so that no more than 20% of substrate was dephosphorylated

Release of $^{32}P_i$ from protein substrates was also measured by isobutanol/benzene extraction of the phosphomolybdate complex (181).

3.5.1 Protein Phosphatase-1

Phosphatase activity was measured at pH 7.4 using the

standard assay mixture following 10 min preincubation with saturating amounts of F_A, 0.1 mM ATP, and 0.5 mM $MgCl_2$.

3.5.2 Protein Phosphatase-2

Activity was measured at pH 8.6 in the presence of the standard assay mixture plus 0.25 µM ^{32}P-protein, 0.1 mM $CaCl_2$, 0.3 µM calmodulin and 20 mM $MgCl_2$. When other divalent cations (1 mM) were substituted for $MgCl_2$, the reaction was performed at pH 7.4 in the absence of DTT.

3.5.3 p-Nitrophenyl Phosphatase

Acid and alkaline phosphatase activities were measured by the release of PNP from PNPP. For acid phosphatase the standard assay mixture (0.5 ml) contained 50 mM Na-Acetate, pH 5.0, 1 mM DTT, 2 mg/ml BSA and 4 mM PNPP. Alkaline phosphatase activity was measured with 50 mM Tris-HCl, pH 8.6, 1 mM DTT, 2 mg/ml BSA, 20 mM $MgCl_2$ and 20 mM PNPP. The reaction was initiated by the addition of enzyme, incubated at 30°C, and terminated by the addition of 0.5 ml 1 M Na_2CO_3. The absorbance at 410 nm of the mixture was measured spectrophotometrically and compared to that of a control mixture lacking added enzyme. The extinction coefficient for p-nitrophenolate anion: 1.75×10^4 M^{-1} cm^{-1}.

One unit of acid or alkaline phosphatase activity was defined as the amount of enzyme catalyzing the release of 1

μmol P_i/min at 30°C.

3.6 Polyacrylamide Gel Electrophoresis

Electrophoresis on 7.5% polyacrylamide gels was carried out at acidic, neutral, or alkaline pH (182-184). Protein was located with Coomassie brilliant blue or by a silver stain (185). For localizing phosphatase activity, gels were transversely sliced into 1 mm sections and each slice was placed in 0.1 ml of 50 mM Tris-HCl, pH 7.0, 10 mM 2-mercaptoethanol, 2 mg/ml BSA, and 10% glycerol for extraction of the enzymatic activity. Alkaline phosphatase activity staining was done in 50 mM Tris-HCl, pH 8.6, 100 mM $MgCl_2$, 1 mM DTT, 20 mM PNPP, and 0.2 M $CaCl_2$. After incubating at 30°C for a suitable length of time, a band of white calcium phosphate appeared on the gel, giving the location of the enzymatic activity.

Polyacrylamide gel electrophoresis in the presence of SDS was carried out according to Laemmli (186). ^{32}P-labeled proteins were located by autoradiography of the dried gels.

3.7 Phosphoamino Acid Analysis

^{32}P-proteins were hydrolyzed in 6 N HCl for 2 h and subjected to thin-layer electrophoresis at pH 3.5 according to the procedure of Hunter and Sefton (24).

3.8 Subcellular Fractionation

Procedures used for the homogenization and fractionation of bovine cerebellum were adapted from the studies of Maeno and Greengard (187,188). Fresh bovine brains were rinsed in cold 0.9% NaCl, the outer meningeal and vascular tissue was removed and 0.5 g sections of the outer cortex were combined from four different cerebella, yielding a total of 2.0 g. The tissue was homogenized in 10 volumes of 0.32 M sucrose and 4 mM Tris-HCl, pH 7.4, with 12 up-and-down strokes at 1000 rpm in a glass homogenizer using a Teflon pestle of 0.15 mm clearance. All steps in the fractionation were carried out at $4^{o}C$. After dilution in the same buffer to 30 ml, the homogenate was centrifuged twice at 1000 x g for 10 min and the pellets were combined to form a P1 fraction (nuclei, plasma membranes, cell debris). The supernatant was then centrifuged for 15 min at 14,000 x g yielding a P2 fraction (mitochondria, plasma membranes). The remaining supernatant was then centrifuged for 60 min at 210,000 x g yielding the P3 (microsomes) and S (cell sap) fractions. The pellets were resuspended using a glass homogenizer with a ground glass pestle. All the fractions were diluted in the same Tris/sucrose buffer described above.

3.9 Other Procedures

Protein concentrations were determined by the method of Lowry *et al* (189) or Bradford (190). BSA was used as a standard.

RESULTS

4.1 Phosphotyrosyl-Protein Phosphatase Activity Associated With Acid Phosphatases

Alkaline phosphatases have been shown to dephosphorylate (P)Tyr-proteins faster than (P)Ser/Thr-proteins (156). Because acid phosphatases are also active toward aromatic phosphoesters which resemble phosphotyrosine, these enzymes may also selectively dephosphorylate (P)Tyr-proteins. Toward this end, the author examined (P)Tyr-protein phosphatase activity from extracts of human prostate; a rich source of acid phosphatase (113). [1]

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1. When the author began this project in 1981, little was known about Tyr-protein kinases, and still less about the corresponding phosphatases. Many of the author's early experiments were limited by technical factors which were overcome as knowledge in this area evolved. For example, initially the available Tyr-protein kinase ($pp60^{\underline{v\text{-}src}}$) was difficult to purify and had limited catalytic capabilities. Therefore, (P)Tyr-protein substrates could only be produced in small quantities, and phosphatase activities were assayed using nanomolar concentrations of ^{32}P-Tyr-substrate. Subsequently, it became possible to obtain a kinase (the EGF receptor/kinase) in quantities sufficient to produce phosphorylated substrates at levels similar to those used in most studies of protein phosphatases. The author employed these more concentrated substrates in his later studies, and was able to obtain kinetic data and other information which had previously been unattainable. Although it would have been interesting to restudy in detail various phosphatases using these more concentrated substrates, by this time, the information gained by this and other laboratories {cont}

4.1.1 Human Prostatic Acid Phosphatase

Co-purification of Phosphotyrosyl-Protein Phosphatase with Acid Phosphatase from Prostate Gland -- Using ^{32}P-Tyr-IgG and PNPP as substrates, the phosphatase activities in the crude homogenate, the 100,000 x g supernatant and pellet derived from prostate gland were examined. Both the ^{32}P-Tyr-IgG and PNP phosphatase activities in each of these fractions exhibited an optimum at pH 5.0, were inhibited about 90% by 20 mM L(+)-tartrate, and were not affected by 1 mM EDTA or 10 mM $MgCl_2$. The results indicate that the predominant ^{32}P-Tyr-IgG phosphatase activity in prostate gland may be identical or related to acid phosphatase. To explore this possibility, purification of ^{32}P-Tyr-IgG phosphatase by a scheme based on the method designed for the purification of acid phosphatase from human prostate gland (114) was carried out. During the purification process, no

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1. (cont) indicated that the protein phosphatases (on which much of the author's early work was done) were unlikely to play more than a minor role in dephosphorylating (P)Tyr-proteins. Instead, efforts were focused on characterizing what appears to be the major enzymatic species responsible for dephosphorylating (P)Tyr-proteins in a representative mammalian tissue (see Results 4.3.3). Where it has been possible to compare results obtained using low and high concentration substrates, the data indicate that the trends observed with the former type of substrate are usually borne out when the latter are used (Results 4.1.1). Therefore, despite the limitations of certain observations, the author is reasonably confident that the overall scheme presented in this thesis has adequate support.

^{32}P-Tyr-IgG phosphatase activity other than that exhibiting acidic pH optimum and sensitivity to inhibition by L(+)-tartrate could be detected. The ^{32}P-Tyr-IgG and the PNP phosphatase activities were found to comigrate on the two DEAE-cellulose chromatographies employed. The purification procedures are summarized in Table 3. The data indicate that the activity ratio between ^{32}P-Tyr-IgG and PNP phosphatase activity is essentially constant after the first DEAE-cellulose chromatography. These two phosphatase activities were purified about 40- and 20-fold, with 14 and 7% recovery, respectively, from the 100,000 x g supernatant.

The ^{32}P-Tyr-IgG and the PNP phosphatase activities in the purified enzyme preparation co-migrated on acidic (Fig. 1) or alkaline (not shown) polyacrylamide gel electrophoresis. It should be noted the high mobility, inactive protein band shown in gel A represents BSA added to the purified enzyme preparation for the purpose of stabilizing the phosphatase activity. SDS-polyacrylamide gel electrophoresis of the enzyme preparation resulted in a single protein staining band corresponding to M_r 50,000 (Fig. 1). Gel filtration of the enzyme preparation on Sephacryl S-200 yielded a single protein peak corresponding to M_r 100,000, which contained both the ^{32}P-Tyr-IgG and the PNP phosphatase activities (not shown). It has been reported that human prostatic acid

phosphatase has an M_r 100,000 and consists of two identical subunits of M_r 50,000 (191-193). Thus, the present results indicate that the ^{32}P-Tyr-IgG phosphatase purified from prostate gland is indeed identical to acid phosphatase. This conclusion is further supported by the observation that these two enzymatic activities exhibit identical thermal stabilities at either 55° or 60°C (Fig. 2).

Time Course and Effects of Enzyme Concentration on Dephosphorylation of ^{32}P-Tyr-IgG -- The activity of prostatic phosphatase towards ^{32}P-Tyr-IgG (0.4 nM) is linearly proportional to incubation time up to 20 min (Fig. 3). At this point, about 20% of the substrate is dephosphorylated. When the incubation is carried out for 10 min, the reaction rate is linearly proportional to the enzyme concentration up to about 0.25 µg/ml. The data indicate that the dephosphorylation reaction follows normal enzyme kinetics despite the use of substrate concentrations in the nanomolar range.

Effects of Inhibitors -- It has been reported that molybdate, vanadate and L(+)-tartrate are potent inhibitors of human prostatic acid phosphatase because their structures resemble the trigonal bipyramidal transition state structure of the enzyme (111). Inorganic phosphate is a less potent inhibitor since it has a tetrahedral structure (111). As

shown in Table 4, all these compounds inhibit both the ^{32}P-Tyr-IgG and the PNP phosphatase activities while D(-)-tartrate is ineffective. The ^{32}P-Tyr-IgG phosphatase is more sensitive to inhibition by these compounds. In order to further explore this phenomenon, the effects of L(+)-tartrate concentrations on the enzymatic activities were examined. The concentration of L(+)-tartrate required for inhibiting 50% of the ^{32}P-Tyr-IgG and the PNP phosphatase activities are 0.07 and 0.7 mM, respectively (Fig. 4a). This phenomenon may simply reflect the fact that the concentration of ^{32}P-Tyr-IgG (0.4 nM) employed in the reaction mixtures is far below saturating concentration (Fig. 4b, insert). On the other hand, the concentration of PNPP (4 mM) employed is about 40-times higher than the K_m value (0.1 mM). The presence of lower substrate concentrations will generally result in an apparent higher inhibition of an enzymatic activity by an inhibitor of competitive type. Table 4 also shows that $ZnCl_2$ and PP_i are inhibitory but are less effective than L(+)-tartrate. Again, a greater degree of inhibition is seen towards ^{32}P-Tyr-IgG than PNPP, consistent with the concentrations of these substrates. NaF, at 5 mM concentrations, abolishes both enzymatic activities. Inhibitors -1 and -2, at concentrations which inhibit more than 90% of the activity of protein phosphatase-1, show slightly stimulatory

effects.

If the dephosphorylation of ^{32}P-Tyr-IgG is catalyzed by acid phosphatase, one would expect PNPP to inhibit this reaction. This is indeed the case. As shown in Fig. 4b, PNPP inhibits the ^{32}P-Tyr-IgG phosphatase activity in a concentration-dependent manner and the concentration for 50% inhibition is about 0.8 nM.

Substrate Specificity -- Due to the limitation of catalytic capability of pp60$^{v\text{-}src}$ kinase activity, ^{32}P-Tyr-IgG and ^{32}P-Tyr-casein could be prepared at no higher than nanomolar concentrations. As a result, the substrate specificity of the prostatic enzyme was studied using (P)protein concentrations in the nanomolar range. The relative activity of the enzyme is given in terms of fmols $^{32}P_i$ released per min, at the stated substrate concentrations. As shown in Table 5, the prostatic enzyme is active towards ^{32}P-Tyr-IgG, ^{32}P-Tyr-casein and PNPP at either pH 5.0 or 7.0. However, it shows little activity towards either ^{32}P-Ser-casein, -glycogen synthase b, -phosphorylase a or ^{32}P-Thr-inhibitor-1. In order to clarify whether the lack of activity towards (P)Ser-proteins is merely a reflection of the low substrate concentrations used, assay of acid phosphatase activity with increasing concentrations of ^{32}P-Ser-phosphorylase a to 10 uM was

performed, as well as ^{32}P-Ser-casein and ^{32}P-Thr-inhibitor-1 to 2 μM. The results indicate that acid phosphatase shows no significant activity toward these (P)Ser/Thr-proteins at acidic, neutral, or alkaline pH. In contrast to the prostatic enzyme, bovine cardiac muscle protein phosphatase-1, activated by F_A and ATP-Mg, dephosphorylates (P)Ser- and (P)Thr-proteins without significant activity towards (P)Tyr-proteins or PNPP at pH 7.0 (Table 5) or at pH 5.0 and 8.6 (data not shown). It should be noted that the protein phosphatase-1 preparation used in the present studies has a specific activity of about 350 nmol/min/mg when assayed with 10 μM phosphorylase a at pH 7.0. Bovine kidney alkaline phosphatase exhibits higher activity toward (P)proteins at neutral rather than alkaline conditions. It preferentially dephosphorylates (P)Tyr-proteins but also shows significant activity toward (P)Ser- and (P)Thr-proteins.

The apparent high specificity of prostatic acid phosphatase toward (P)Tyr-proteins raises the possibility that other acid phosphatase isozymes may also possess such characteristics. In order to examine this possibility, the activities of several commercial acid phosphatases of human origin and an acid phosphatase from wheat germ were studied. As shown in Table 6, all these enzymes exhibit higher activity toward ^{32}P-Tyr-casein than ^{32}P-Ser-casein.

Table 3. Co-purification of (P)Tyr-protein and acid phosphatase activities from human prostate gland The phosphatase activities were purified from prostate gland (2.26 g) obtained from a patient with benign hypertrophy. At each stage of the purification, the enzymatic activities were determined at pH 5.0 with ^{32}P-Tyr-IgG (1.56 nM) or PNPP (Experimental Procedures 3.5 and 3.5.3).

[a] Units are defined as fmol/min for ^{32}P-Tyr-IgG and nmol/min for PNP phosphatase activities.

Fraction	Total protein	Specific activity on		
		^{32}P-Tyr-IgG (A)	PNPP (B)	(A/B)
	mg	units/mg[a]		
1. 100,000 x g supernatant	27	14	67	(0.21)
2. First DEAE-cellulose	4.3	119	312	(0.38)
3. Acid-ammonium sulfate fractionation	0.43	409	1007	(0.41)
4. Second DEAE-cellulose	0.1	553	1294	(0.43)

Table 4. Effects of various compounds on the prostatic (P)Tyr-protein and acid phosphatase activities The phosphatase activities were measured at pH 5.0 with ^{32}P-Tyr-IgG (1.56 nM) or PNPP (4 mM) (Experimental Procedures 3.5 and 3.5.3) in the absence or presence of effectors as indicated.

Addition	Activity on	
	^{32}P-Tyr-IgG	PNPP
	%	
None	100	100
Molybdate, 1 µM	9	7
10 µM	0	0
Vanadate, 10 µM	21	54
100 µM	6	5
L(+)-Tartrate, 5 mM	4	9
D(-)-Tartrate, 5 mM	118	111
NaF, 5 mM	0	0
KP_i, 5 mM	20	93
$NaPP_i$, 5 mM	81	123
$ZnCl_2$, 10 µM	100	112
100 µM	45	104
Inhibitor-1, 10 nM	110	124
Inhibitor-2, 30 nM	173	138

Table 5. Substrate specificity of human prostatic acid phosphatase and bovine kidney alkaline phosphatase The human prostatic acid phosphatase purified in this study was measured with PNPP (4 mM) or ^{32}P-protein at the indicated concentrations. Bovine kidney alkaline phosphatase was measured with the same incubation mixture except that the concentration of PNPP was 20 mM and that $MgCl_2$ (20 mM) was also included.

[a] Activity units are defined as fmol/min for ^{32}P-proteins and μmol/min for PNPP

^{32}P-protein	Activity of phosphatases at			
	pH 5.0	pH 7.0		pH 8.6
	human acid	human acid	bovine alkaline	bovine alkaline
	units/mg[a]		milliunits/mg	
^{32}P-Tyr-IgG (0.55 nM)	318	33	61	5.2
^{32}P-Tyr-casein (0.25 nM)	61	6	31	7.2
^{32}P-Ser-casein (0.65 nM)	2.5	0.7	17	2.4
^{32}P-Ser-phosphorylase (0.57 nM)	1.0	0.1	0.6	1.6
^{32}P-Ser-glycogen synthase (0.36 nM)	0.1	0	7.1	2.6
^{32}P-Thr-inhibitor-1 (1.1 nM)	1.0	0.8	2.9	0.6
PNPP	1003	688	87	430

Table 6. Specificity of acid phosphatase isozymes The phosphatase activities were measured at pH 5.0 with ^{32}P-Tyr-casein (0.40 nM), ^{32}P-Ser-casein (0.44 nM) or PNPP (20 mM). Activity units are defined as fmol/min for ^{32}P-proteins and nmol/min for PNPP.

Acid phosphatase	Activity on		
	^{32}P-Tyr-casein	^{32}P-Ser-casein	PNPP
	U/ml		
Seminal fluid, band 2	16.4	0.30	501
Spleen, band 3	2.5	0.35	102
Leukocyte, band 4	13.6	2.65	414
Serum, band 5a	1.6	0.25	28
Serum, band 5b	2	0.20	6
Wheat germ	50	21	459

Fig. 1. Polyacrylamide gel electrophoresis of purified (P)Tyr-protein phosphatase from human prostate gland. Purified phosphatase (3.4 μg) mixed with BSA (6.6 μg) was loaded on 7.5% polyacrylamide gels (182). Following electrophoresis, one gel was sliced into 1-mm sections and assayed at pH 5.0 for the activity toward 0.4 nM ^{32}P-Tyr-IgG (●-●) and PNPP (○-○); the other, gel A, was stained for protein. In separate experiments, the phosphatase was electrophoresed on 10% polyacrylamide gels in the presence of SDS. Gel B represents the protein staining pattern of the enzyme plus BSA. Gel C, protein molecular weight standards: phosphorylase b (92.5 kDa), BSA (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa), and lysozyme (14.4 kDa)

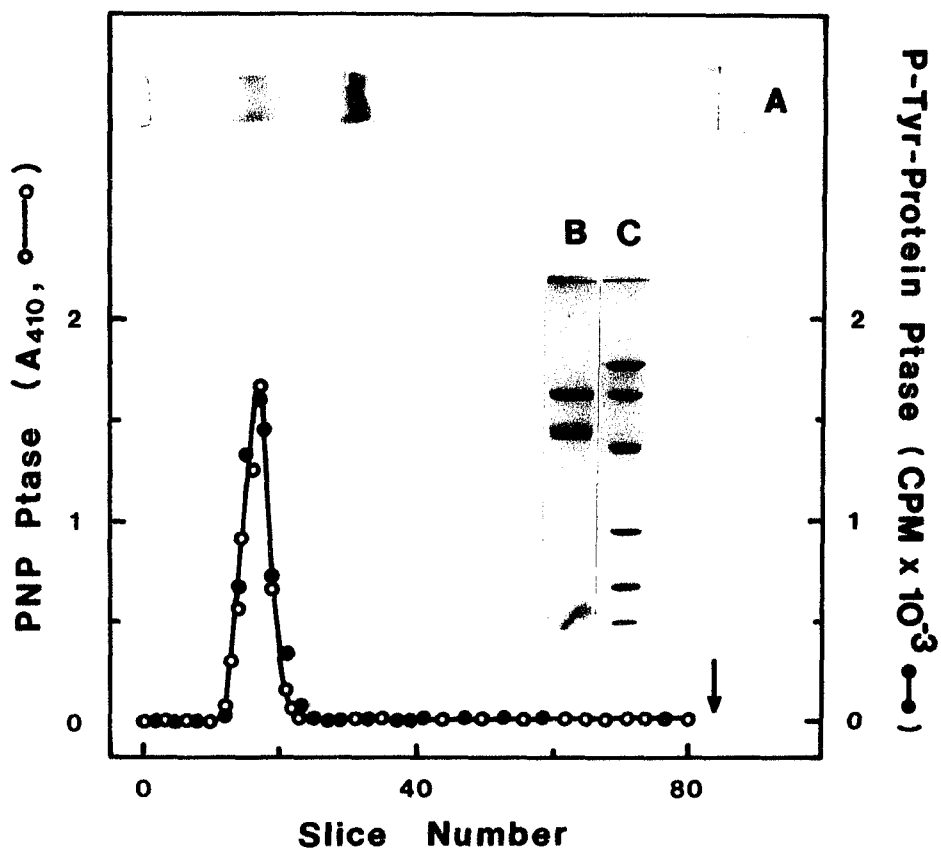


Fig. 2. Thermostability of prostatic (P)Tyr-protein phosphatase. The purified (P)Tyr-protein phosphatase was incubated at 55°C and 60°C with 20 mM Tris/HCl, pH 7.4, 0.2% BSA. At the indicated time intervals, aliquots were withdrawn and assayed at pH 5.0 for activity toward 0.4 nM ^{32}P-Tyr-IgG (●-●) or PNPP (○-○)

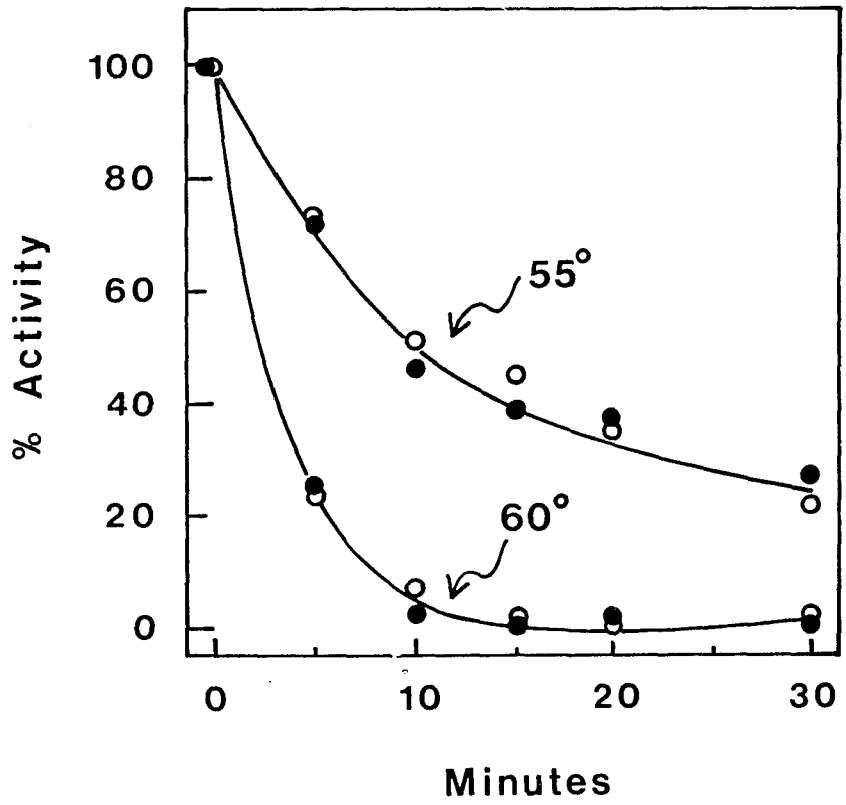


Fig. 3. Time course and effects of phosphatase concentration on dephosphorylation of ^{32}P-Tyr-IgG. For study of the time course, the reaction was carried out at 37°C in a volume of 250 µl containing 50 mM Na Acetate, pH 5.0, 1 mM DTT, 0.4 mg/ml BSA, 0.4 nM ^{32}P-Tyr-IgG and 0.124 µg/ml phosphatase. At the indicated time intervals, aliquots of 25 µl were withdrawn for determination of $^{32}P_i$ released. For study of the effects of enzyme concentration, the reaction was carried out at 37°C for 10 min in a volume of 25 µl containing the same components as described above except that the phosphatase concentration was varied as indicated. The phosphatase activity is expressed as the percentage of dephosphorylation of ^{32}P-Tyr-IgG for either the time course (● - ●) or enzyme concentration curve (○ - ○)

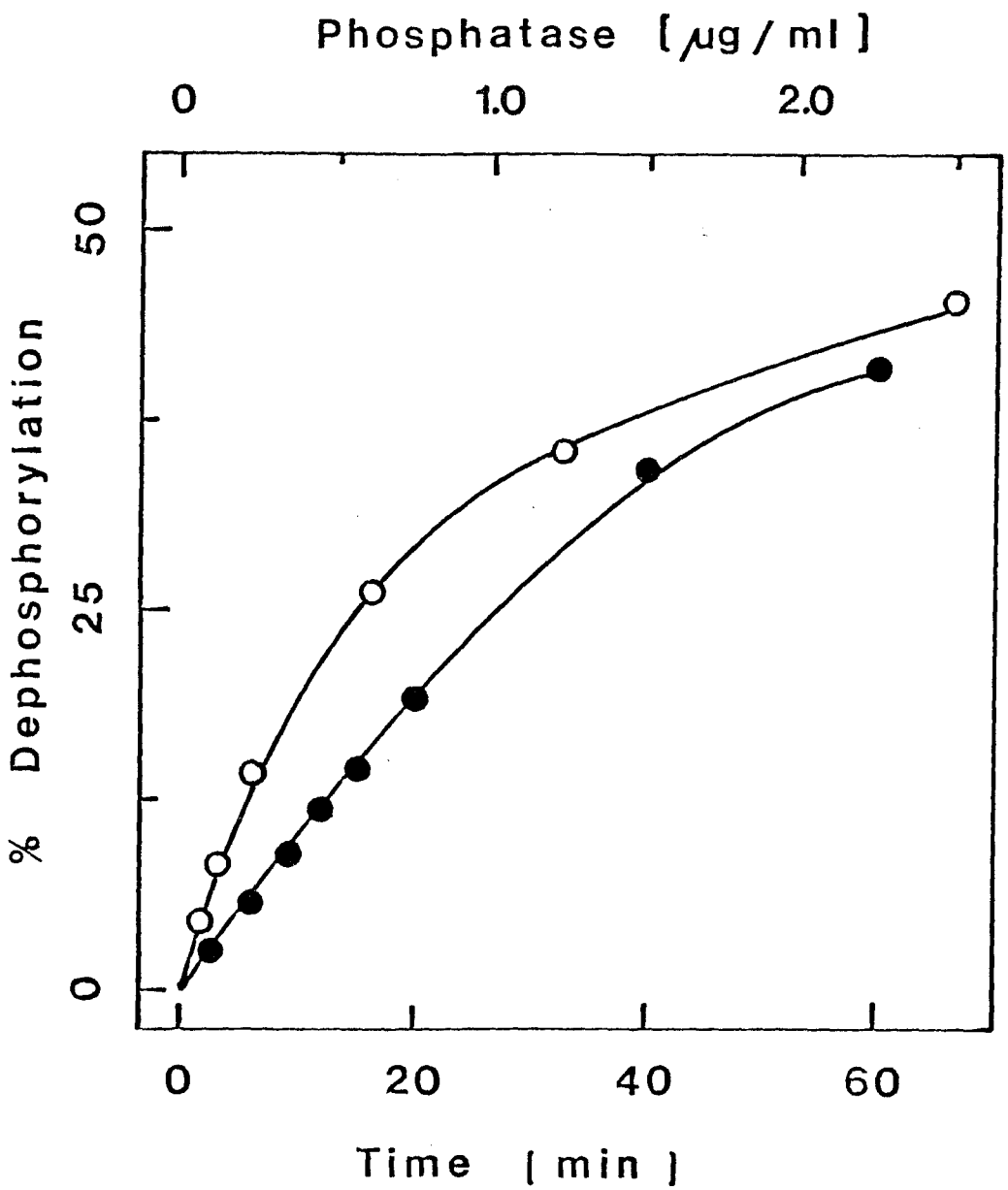
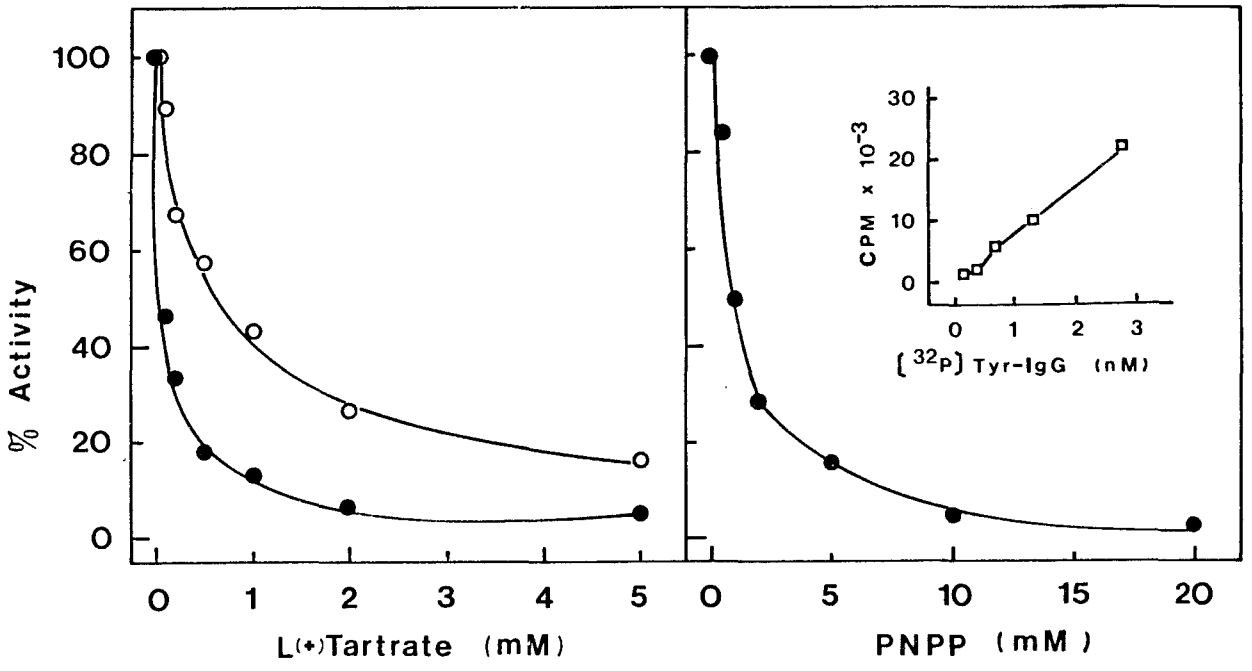


Fig. 4. Effects of L(+)-tartrate, PNPP, and ^{32}P-Tyr-IgG concentrations on prostatic (P)Tyr-protein phosphatase. (A) The enzymatic activities were measured with 0.4 nM ^{32}P-Tyr-IgG (●- ●) or 4 mM PNPP (○- ○) in the absence and presence of various concentrations ofL(+)-tartrate as indicated. (B) The activity was meaured with 0.4 nM ^{32}P-Tyr-IgG (●- ●) in the absence and presence of various concentrations of PNPP as indicated. The insert shows the effects of ^{32}P-Tyr-IgG concentrations on the enzymatic activity (□- □) in the absence of effector



4.2 Phosphotyrosyl Protein Phosphatase Activity Associated With Classical Protein Phosphatases

The protein phosphatases types 1-4 have been extensively studied in terms of their structure, regulation, and activity towards various (P)Ser- and (P)Thr-proteins. In order to determine whether these enzymes might also function as (P)Tyr-protein phosphatases, and, if so, to examine the characteristics of this activity, a systematic study of this activity was undertaken.

4.2.1 Protein Phosphatase-1

M_r 61,000 type 1 protein phosphatase from bovine heart, assayed in the presence of the activators F_A, ATP, and Mg^{2+}, does not display detectable activity towards ^{32}P-Tyr-IgG or ^{32}P-Tyr-casein at neutral or alkaline pH. Because this enzyme elutes from DEAE-cellulose at a similar salt concentration as (P)Tyr-protein phosphatase Y-2, an enzyme stimulated by chelating agents (see Results 4.3.3), phosphatase-1 was also assayed in the presence of EDTA in order to elicit any latent (P)Tyr-protein phosphatase activity. However, the enzyme was inactive under these conditions, in the presence or absence of F_A and ATP (not shown).

4.2.2 Protein Phosphatase-2 (Calcineurin)

Synergistic Activation of the Calcineurin Phosphatase Activity Towards ^{32}P-Tyr-casein by Ca^{2+}, Calmodulin and Mg^{2+} -- Purified bovine brain calcineurin is able to dephosphorylate ^{32}P-Tyr-casein (Table 7). The addition of Ca^{2+} or calmodulin alone has little effect on this activity, but Ca^{2+}/calmodulin stimulates the enzymatic activity. Mg^{2+} alone is slightly more effective than Ca^{2+} plus calmodulin. The Mg^{2+} activating effect is not significantly affected by calmodulin alone, but is stimulated by Ca^{2+} alone more than 5-fold. Full expression of the phosphatase activity requires the simultaneous presence of Ca^{2+}, calmodulin and Mg^{2+}. The level of activity achieved in the presence of these three effectors is far in excess of the sum of the activity observed when these agents are added alone. The data indicate that (a) the synergistic action of Ca^{2+} and Mg^{2+} leads to partial activation (about 15% of the full activity) of the enzyme, and, (b) the synergistic action of Ca^{2+}, calmodulin and Mg^{2+} leads to full activation of the phosphatase.

Divalent Cation Specificity -- Besides Mg^{2+}, all transition metal ions examined (Ni^{2+}, Mn^{2+}, and Co^{2+}) may serve to activate calcineurin (Table 8). Zn^{2+}, Cu^{2+}, and Fe^{2+} are ineffective. The activating effects of these

transition metal ions differ from that of Mg^{2+} in several respects: (a) In the absence of Ca^{2+} and calmodulin, the transition metal ions activate the enzyme to a much higher level that Mg^{2+} does. However, Ca^{2+}/calmodulin stimulates the Mg^{2+}-supported activity to a much greater extent (25- to 40-fold) than the transition metal ion-supported activities (4- to 7-fold). (b) The Mg^{2+}-supported activity is higher at pH 8.6 than 7.4, while the opposite is true for the transition metal ion-supported activity (not shown). As shown in Table 8, when measured at their respective optimal conditions in the presence of Ca^{2+}/calmodulin, the order of effectiveness of these divalent cations is $Ni^{2+} > Mn^{2+} > Mg^{2+} > Co^{2+}$.

Kinetic Parameters -- The saturation curve of Mg^{2+}-supported activity for ^{32}P-Tyr-casein follows Michaelis-Menten kinetics, either in the absence or presence of Ca^{2+}/calmodulin (Fig. 5). In the presence of Mg^{2+}, the K_m and V_{max} of calcineurin phosphatase activity towards ^{32}P-Tyr-casein is approximately 2.2 μM and 0.4 nmol/min/mg, respectively. The addition of Ca^{2+}/calmodulin decreases the K_m to 0.6 μM, while increasing the V_{max} to 4.6 nmol/min/mg. This 11-fold increase in V_{max} with a smaller change in K_m, observed when Ca^{2+}/calmodulin are added to divalent cation-stimulated calcineurin, is similar to results obtained when PNPP is used as substrate (144). Similar

experiments performed with ^{32}P-Ser-casein and ^{32}P-Thr-inhibitor-1 indicate that, in the presence of Mg^{2+} and Ca^{2+}/calmodulin, the K_ms for these substrates are slightly higher than that measured for ^{32}P-Tyr-casein (1.0 and 4.0 μM) but that these substrates are dephosphorylated by calcineurin at a much faster rate than ^{32}P-Tyr-casein (V_{max} = 121 and 258 nmol/mg/min, respectively).

In the presence of Ca^{2+}/calmodulin, the Mg^{2+} stimulation of the calcineurin phosphatase activity towards ^{32}P-Tyr-casein is concentration dependent and follows Michaelis-Menten kinetics (not shown). The K_a value for Mg^{2+} is about 5 mM. Thus, the 20 mM $MgCl_2$ used in routine assays represents a saturating concentration for the phosphatase reaction.

Comigration of Ca^{2+}/Calmodulin- and Mg^{2+}-Stimulated (P)Tyr-Protein Phosphatase Activity on Polyacrylamide Gel Electrophoresis -- As shown in Figure 6, polyacrylamide gel electrophoresis of the calcineurin preparation results in one major and two minor protein bands (Gel A). These co-migrate with the major and minor peaks of phosphatase activity. The major active species, which represents more than 90% of the enzymatic activity recovered from the gel, is partially activated by Mg^{2+} alone or Ca^{2+} plus calmodulin, and is fully activated by Ca^{2+}, calmodulin, and

Mg^{2+}. Both of the two minor protein bands are active. The one with lower mobility than the major active species can be stimulated by Mg^{2+} but this Mg^{2+}-supported activity cannot be further augmented by Ca^{2+}/calmodulin. By contrast, the other minor species remains responsive to all three effectors. These minor active species may derive from calcineurin by limited proteolysis (140). Gel B shows that two major protein bands are obtained from SDS-polyacrylamide electrophoresis of calcineurin. These represent the A-(M_r 61,000) and B-(M_r 15,000) subunits of calcineurin, respectively. The data indicate that the (P)Tyr-protein phosphatase activity is an intrinsic property of calcineurin, and that the observed effects of divalent cation cannot be attributed to a contaminating enzyme.

Dephosphorylation of A-431 Cell Membrane Proteins by Calcineurin -- In order to demonstrate that calcineurin possesses protein phosphatase activity toward a protein known to be phosphorylated at tyrosine in vivo, and that the characteristics of this activity resemble those seen toward the artificial substrate ^{32}P-Tyr-casein, experiments were performed utilizing autophosphorylated A-431 cell membrane proteins as substrates for this enzyme. The phosphorylated proteins are comprised mostly of the M_r 170,000 and 150,000 EGF receptor, and a M_r 25,000 which may represent a proteolytic fragment of the receptor. More than 95% of the

bound radioactivity is incorporated into tyrosine residues (not shown). In the absence of activators, calcineurin is unable to dephosphorylate the membrane phosphoproteins (Fig. 7, lane B). The addition of Ca^{2+}/calmodulin (lane C), Mg^{2+} (lane D), or Ca^{2+} and Mg^{2+} (lane E) partially stimulates the phosphatase activity, while the addition of these Ca^{2+}, calmodulin, and Mg^{2+} together causes nearly complete dephosphorylation of the membrane proteins (lane F). In the absence of added calcineurin, these effectors do not stimulate dephosphorylation (not shown). Thus, the results achieved with autophosphorylated A-431 membrane proteins are qualititatively similar to those observed when ^{32}P-Tyr-casein is used as substrate.

Table 7. Synergistic activation of the calcineurin phosphatase activity by Mg^{2+}, Ca^{2+}, and calmodulin. The enzymatic activity was measured in the presence of 50 mM Tris-HCl, pH 8.6, 0.5 mM DTT, 0.2 mg/ml BSA, 0.5 μM ^{32}P-Tyr-casein, and where present, 50 μM EGTA, 0.1 mM $CaCl_2$, 0.3 μM calmodulin, or 20 mM $MgCl_2$, alone, and in the indicated combinations, for 20 min.

Addition	Phosphatase Activity (nmol/min/mg)
EGTA	0.01
Ca	0.01
calmodulin + EGTA	0
Ca + calmodulin	0.13
Mg + EGTA	0.17
Mg + Ca	0.88
Mg + Ca + calmodulin	5.89
Mg + EGTA + calmodulin	0.18

Table 8. Divalent-cation specificity of the calcineurin phosphatase. The enzymatic activity was assayed with the the indicated divalent cations in the presence of 50 μM EGTA or 0.1 mM Ca^{2+}, 0.3 μM calmodulin, for 20 min as described in "Experimental Procedures" (3.5.2)

Addition	Phosphatase Activity (nmol/min/mg)	
	EGTA	Ca/calmodulin
none	0	0.26
Mg[a]	0.1	2.6
Mg[b]	0.1	3.9
Ni	4.5	17.6
Mn	1.9	8.2
Zn	0.0	0
Cu	0.04	0.04
Co	0.4	3.3
Fe	0.03	0
Ca	0.01	0.19

[a] Assayed at pH 7.4

[b] Assayed at pH 8.6

Fig. 5. Effects of Ca^{2+}/calmodulin on the kinetics of calcineurin phosphatase activity towards ^{32}P-Tyr-casein. The initial reaction rate was measured at 30° C in an incubation volume of 50 µl containing 50 mM Tris-HCl, pH 8.6, 0.2 mg/ml BSA, 0.5 mM DTT, 20 mM $MgCl_2$, and various ^{32}P-Tyr-casein concentrations in the presence of 50 µM EGTA (○-○) or 0.1 mM Ca^{2+} and 0.3 uM calmodulin (●-●). The insert shows a double reciprocal plot of the same data.

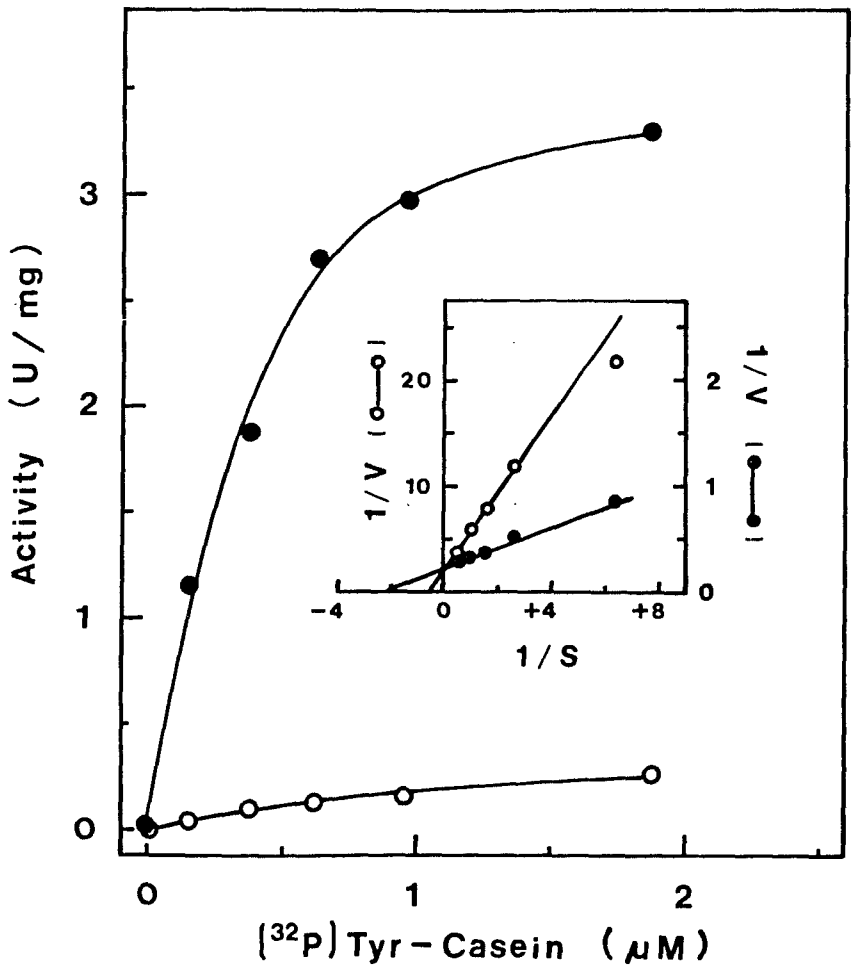


Fig. 6. Polyacrylamide gel electrophoresis of calcineurin. 25 μg of enzyme was loaded onto two lanes of a 7% polyacrylamide slab gel (25). Following electrophoresis, one lane (gel A) was stained for protein and the second was sliced and assayed for ^{32}P-Tyr-protein phosphatase activity in the presence of no effector (■ - ■), Ca^{2+}/calmodulin (□ - □), Mg^{2+} (O - O), and Mg^{2+}, Ca^{2+}/calmodulin (● - ●), as described in "Experimental Procedures" (3.5.2). The arrow indicates the dye front. Gel B shows the protein staining pattern of the enzyme following electrophoresis on a 10% polacrylamide gel in the presence of SDS. The standards are phosphorylase b, BSA, ovalbumin, carbonic anhydrase, trypsin inhibitor, and lysozyme.

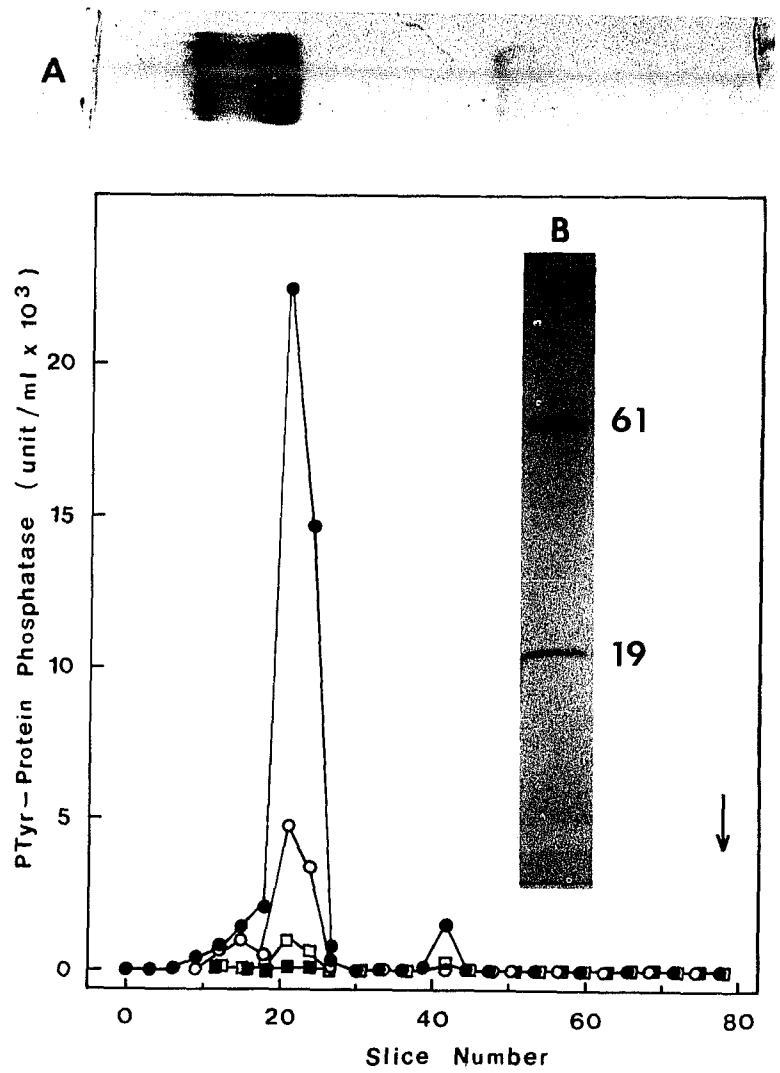
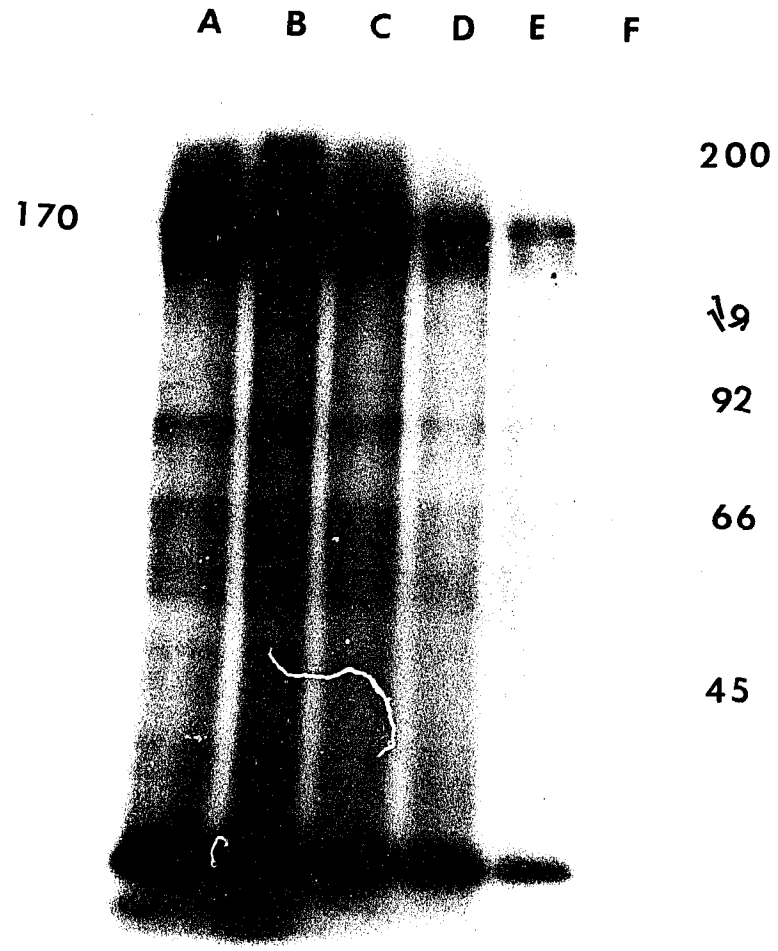


Fig. 7. Dephosphorylation of A-431 membrane proteins by calcineurin. A-431 membrane proteins phosphorylated at tyrosine residues (1 nM) were incubated in the absence (Lane A) and presence of calcineurin (50 µg/ml) with the following effectors: no addition (B), 0.1 mM Ca^{2+} and 0.3 µM calmodulin (C), 20 mM Mg^{2+} (D), Ca^{2+} and Mg^{2+} (E), or Ca^{2+}/calmodulin plus Mg^{2+} (F) at 30°C for 40 min as described in "Experimental Procedures" (3.5.2). The reactions were terminated with SDS sample buffer, followed by electrophoresis on an 8% SDS-polyacrylamide gel. Following electrophoresis, the gel was stained, destained, dried, and exposed to X-ray film for 48 hrs. An autoradiograph of the dried gel is shown. The standards are myosin heavy chain, b-galactosidase, phosphorylase b, BSA, and ovalbumin.



4.2.3 Protein Phosphatase-3

Activation of ^{32}P-Tyr-IgG and PNP Phosphatase Activity by Mg^{2+} -- As shown in Fig. 8, the M_r 95,000 phosphatase preparation from bovine heart is active towards ^{32}P-Ser-phosphorylase a in the absence of added divalent cation, but shows little, if any, activity towards either ^{32}P-Tyr-IgG or PNPP. When Mg^{2+} is added to the assay mixture, the enzymatic activity toward ^{32}P-Ser-phosphorylase a is slightly inhibited, while those toward ^{32}P-Tyr-IgG and PNPP become activated in a concentration-dependent manner. Both the saturation curves and the K_m values for Mg^{2+} (about 13 mM) for these two activities are similar.

pH Optima -- Fig. 9 shows that the pH activity profiles of the ^{32}P-Tyr-IgG and PNP phosphatase activities are similar, and that both activities have an optimum around pH 8.5-9.0. In contrast, phosphorylase phosphatase activity, measured in the presence of 2 mM EDTA (Fig. 9) or 20 mM $MgCl_2$ (not shown), has an optimal pH around 7.5. It should be noted that, in the absence of Mg^{2+}, no activity can be detected towards ^{32}P-Tyr-IgG (Fig. 9) or PNPP (not shown) throughout the range of pH studied.

Thermal Stability -- As shown in Fig. 10, the phosphorylase phosphatase activity is relatively stable at 40°C. Both the ^{32}P-Tyr-IgG and the PNP phosphatase

activities, however, are rapidly inactivated in a parallel fashion at this temperature. Within 30 min, the phosphatase becomes completely inactive towards these substrates.

Effects of Phosphatase Inhibitors -- Fig. 11 shows the effects of increasing P_i ccncentrations on phosphorylase, ^{32}P-Tyr-IgG, and PNP phosphatase activities. Although all these activities are inhibited by millimolar levels of P_i, the phosphatase activities towards ^{32}P-Tyr-IgG and PNPP are inhibited to a much greater extent than the phosphorylase phosphatase activity. Moreover, the degree of inhibition of ^{32}P-Tyr-IgG and PNPP dephosphorylation is nearly identical at all levels of P_i concentration. The concentrations of P_i required for 50% inhibition of phosphatase activity is approximately 1 mM in the cases of ^{32}P-Tyr-IgG and PNPP and approximately 7 mM in the case of ^{32}P-Ser-phosphorylase a.

Table 9 shows the effects of various compounds on the activities of the M_r 95,000 phosphatase preparation from bovine cardiac muscle and of commercially obtained alkaline phosphatase form bovine kidney. The M_r 95,000 preparation exhibits markedly different degrees of sensitivity to Zn^{2+}, EDTA, and F^-, when assayed against ^{32}P-Ser-phosphorylase a on the one hand, and ^{32}P-Tyr-IgG and PNPP on the other. In general, the ^{32}P-Tyr-IgG and PNP phosphatase activities are more sensitive to inhibition by Zn^{2+}, EDTA, and F^-, whereas

the phosphorylase phosphatase activity is more sensitive to inhibition by PP_i. When assayed in the presence of each effector, bovine kidney alkaline phosphatase behaves similarly toward ^{32}P-Tyr-IgG and PNPP and this behavior does not resemble that of the cardiac enzyme. For example, both the P-Tyr-IgG and the PNP phosphatase activities associated with the kidney enzyme are slightly stimulated in the presence of 50 μM Zn^{2+}, and are inhibited about 40% in the presence of 50 mM F^-. By contrast, these two activities associated with the cardiac enzyme are inhibited about 30 and 99% in the presence of the same concentrations of Zn^{2+} and F^-, respectively. Table 9 also shows that PNPP is a potent inhibitor of the ^{32}P-Tyr-IgG phosphatase activity associated either with the cardiac or the kidney phosphatase. Furthermore, the ^{32}P-Tyr-IgG phosphatase activity is more sensitive to inhibition by PNPP than the phosphorylase phosphatase activity in the cardiac muscle enzyme preparation.

Comigration of the ^{32}P-Tyr-IgG and the PNP Phosphatase Activities on Polyacrylamide Gel Electrophoresis -- Previous studies have demonstrated that the PNP phosphatase activity activity copurifies with the M_r 35,000 phosphatase (149) and the M_r 95,000 phosphatase which contains an M_r 35,000 catalytic entity (150). This laboratory has examined the ^{32}P-Tyr-IgG phosphatase activity in the processes of

purification of the M_r 95,000 phosphatase from bovine heart and the M_r 35,000 enzyme from rabbit liver (147). The results indicate that the ^{32}P-Tyr-IgG phosphatase activity copurifies with the PNP and phosphorylase phosphatase activities throughout various separation processes including ammonium sulfate fractionation, ethanol treatment, DEAE-cellulose and gel filtration chromatographies, and polyacrylamide gel electrophoresis. Fig. 13 shows the results of polyacrlyamide gel electrophoresis of a typical M_r 95,000 phosphatase preparation. The data indicate that the enzymatic activities are separated into a single active peak of low mobility and doublet active peaks of high mobility. The enzymatic activity towards ^{32}P-Tyr-IgG coincides with those towards PNPP and ^{32}P-Ser-phosphorylase a in either the low mobility or the high mobility doublet bands. The activity profile shown in Fig. 13 reflects the fact that the highly purified M_r 95,000 phosphatase tends to undergo partial dissociation on polyacrylamide gel electrophoresis (150). As previously reported (150), when the proteins in the low mobility band are extracted from the gel and re-electrophoresed on polyacrylamide in the presence of SDS, two protein bands, corresponding to M_r 63,000 and 35,000, are observed. Similar experiments on the high mobility doublet active bands result in a single protein band of M_r 35,000 on SDS-gel electrophoresis. It is

believed that the M_r 95,000 phosphatase consists of a catalytic subunit of M_r 35,000 and a noncatalytic subunit of M_r 63,000 (150). Regardless of the precise subunit composition of this enzyme, the present results clearly demonstrate that the activity towards ^{32}P-Tyr-IgG is tightly associated with those toward PNPP and ^{32}P-Ser-phosphorylase a.

Substrate Specificity -- In order to gain more understanding concerning the specificity of the phosphatase, ^{32}P-proteins specifically labeled at tyrosine, serine, or threonine residues were used as substrates to study the enzymatic activity in various conditions. The concentrations of ^{32}P-proteins used are all in the nanomolar range because of limited availabilty of ^{32}P-Tyr-proteins at the time these experiments were performed. For comparison, the activity of commercial alkaline phosphatase from bovine kidney was also examined. As shown in Table 10, the effects of pH on the activity of the type 3 enzyme toward ^{32}P-Tyr-proteins is dependent on the divalent cation species present in the reaction mixture. In the presence of 0.5 mM Mn^{2+}, the enzyme exhibits much higher activity at pH 7.0 than at 8.6. When 10 mM Mg^{2+} is substituted for Mn^{2+}, the opposite effects of pH on the enzymatic activity are observed. The data also indicate that Mn^{2+} is a more effective activator than Mg^{2+} and that ^{32}P-Tyr-casein is a

better substrate than ^{32}P-Tyr-IgG. It should be noted that, in the absence of added divalent cation, the M_r 95,000 enzyme preparation shows no detectable activity toward ^{32}P-Tyr-proteins or PNPP at either pH 7.0 or 8.6 (not shown). The enzyme, however, dephosphorylates ^{32}P-Ser-casein and ^{32}P-Ser-phosphorylase *a* at similar rates when measured either in the absence or presence of Mg^{2+}. Thus, the catalytic properties of the (P)Tyr-protein phosphatase activity in the M_r 95,000 preparation are indeed distinctly different from those of the (P)Ser-protein phosphatase activity.

When the rates of dephosphorylation of (P)Ser/Thr-proteins and (P)Tyr-proteins by the phosphatase-3 are compared, the data indicate that the former activity is much higher than the latter in all conditions examined (Table 10). In contrast, the kidney alkaline phosphatase preferentially dephosphorylates (P)Tyr-proteins. This enzyme is much more active toward (P)Tyr-proteins at pH 7.0 than at 8.6, while it hydrolyzes PNPP faster under alkaline conditions. These data are consistent with the findings of Swarup *et al* (156).

Table 9. Effects of various agents on cardiac protein phosphatase-3C and kidney alkaline phosphatase activities towards ^{32}P-Ser-phosphorylase, ^{32}P-Tyr-IgG, and PNPP. Aliquots of the cardiac or the kidney phosphatase were incubated with either 10 µM ^{32}P-Ser-phosphorylase, 0.2 nM ^{32}P-Tyr-IgG, or 20 mM PNPP as described in "Experimental Procedures" (3.5 and 3.5.3). Enzymatic activity is given as a percentage of the activity in the absence of additions, each the average of duplicates for three determinations.

Addition	Activity (% control)				
	Phosphatase 3C			Kidney alkaline phosphatase	
	Phos-phory-lase	IgG	PNPP	Phos-phory-lase	IgG	PNPP
$ZnCl_2$, 50 µM	125	78	61	116	149
PNPP, 2 mM	100	34		24	
PNPP, 5 mM	67	19		8	
NaF, 50 mM	34	0.7	1	60	56
EDTA, 5 mM	98	2.5	0	2.4	3.7
PP_i, 2 mM	68	90	137	115	112

Table 10. Substrate specificities of cardiac protein phosphatase-3C and kidney alkaline phosphatase. The activities of the bovine cardiac (P)Ser-protein phosphatase-3C (1 mg/ml) and kidney alkaline phosphatase (2.5 mg/ml) were measured at pH 7.0 or 8.6 as described in "Experimental Procedures" (3.5 and 3.5.3) with various ^{32}P-proteins and PNPP at the indicated concentrations. The cardiac enzyme was assayed in the presence of $MgCl_2$ (10 mM) or $MnCl_2$ (0.5 mM) as indicated. The kidney alkaline phosphatase was measured in the presence of $MgCl_2$ (10 mM). Activity is expressed as fmol/min/ml for phosphoprotein phosphatase and µmol/min/ml for PNP phosphatase.

Substrate	Activity					
	Phosphatase 3C				Kidney alkaline phosphatase	
	Mg^{2+}		Mn^{2+}			
	7.0	8.6	7.0	8.6	7.0	8.6
^{32}P-Tyr-IgG (0.56 nM)	47	154	725	48	150	13
^{32}P-Tyr-casein (0.40 nM)	164	570	1870	690	83	29
^{32}P-Ser-casein (0.44 nM)	4300	6100	26800	19400	12	6
^{32}P-Ser-phosphorylase (0.56 nM)	5920	5799	43071	26837	2	4
^{32}P-Thr-inhibitor-1 (1.12 nM)	1573	24173	119006	42785	7	2
PNPP (4 mM)	0	127	28	47	0	1

Fig. 8. Effects of $MgCl_2$ on the activities of cardiac protein phosphatase-3C. The enzymatic activities toward ^{32}P-Ser-phosphorylase a (Phlase a) (□), ^{32}P-Tyr-IgG (○), and PNPP (×) were measured under standard assay conditions (Experimental Procedures 3.5 and 3.5.3) at pH 7.0, 8.6, and 8.6, respectively. $MgCl_2$ concentrations were varied as indicated

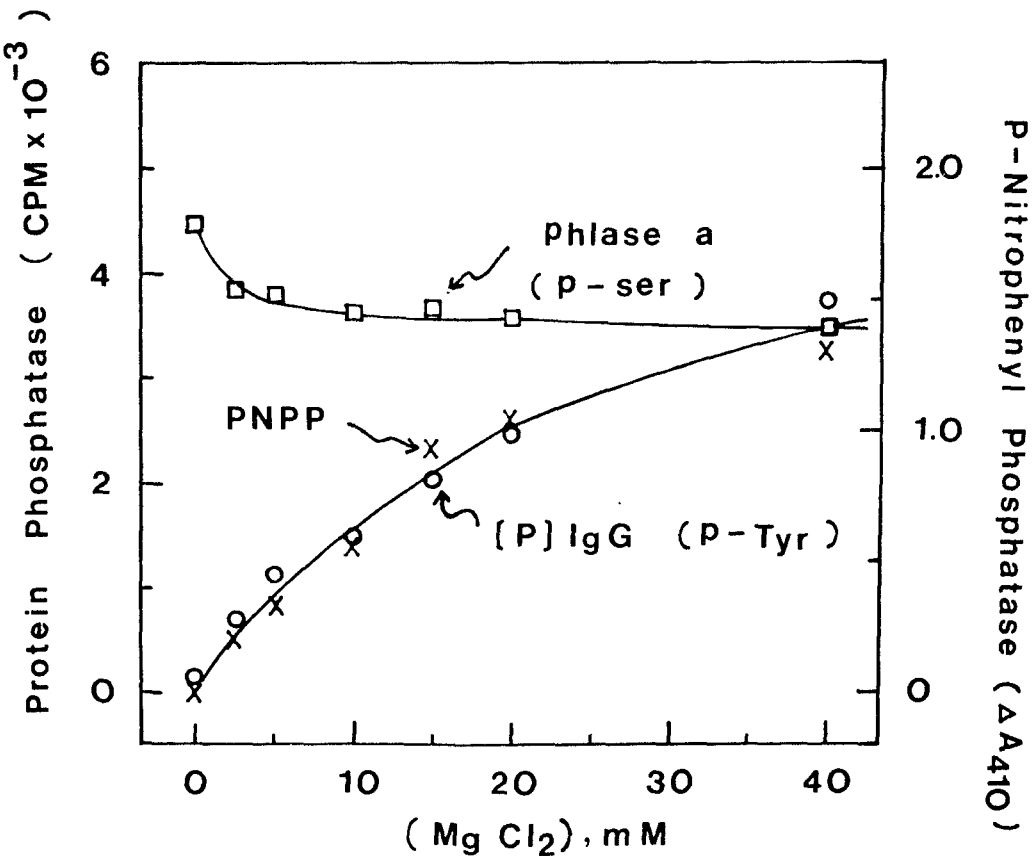


Fig. 9. Effects of pH on the activities of protein phosphatase-3C. The enzymatic activities toward ^{32}P-Ser-phosphorylase a (with 2 mM EDTA, □ , ■), ^{32}P-Tyr-IgG (with 2 mM EDTA, ⊖ , ; 20 mM $MgCl_2$, ○ , ●) and PNPP (with 20 mM $MgCl_2$, △ , ▲) were measured as described in "Experimental Procedures" 3.5 and 3.5.3 except that 50 mM of the following buffers at the indicated pH were used: Tris-HCl (□ , ○ , ⊖ , and △) and imidazole (■ , ● , and ▲)

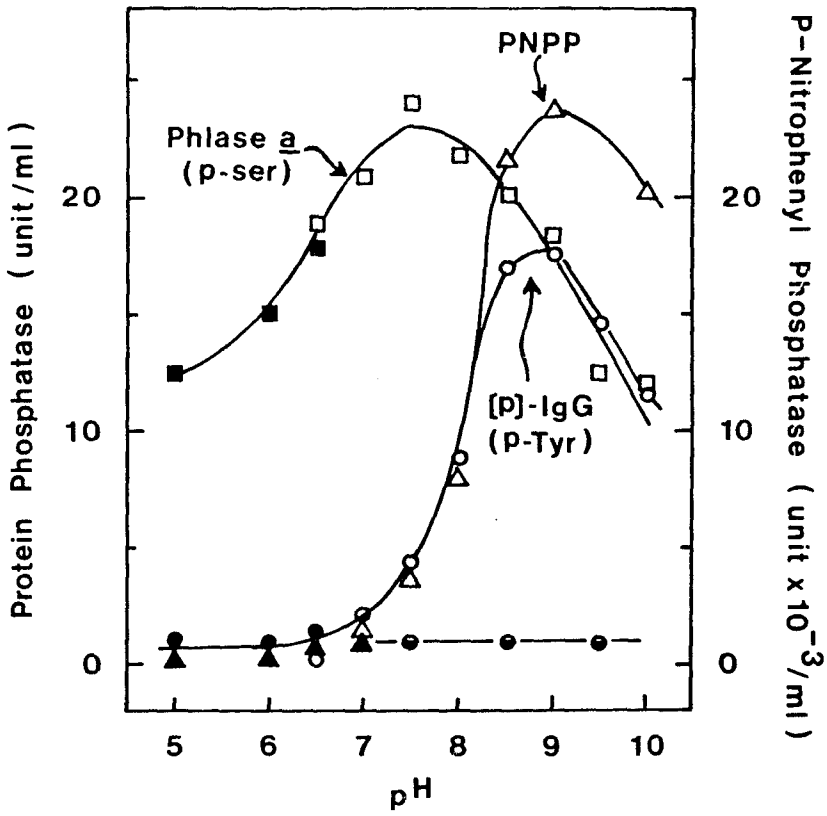


Fig. 10. Thermal stability of cardiac protein phosphatase-3C. The enzyme was preincubated at 40°C in a solution containing 20 mM Tris-HCl, pH 7.4, 2 mM 2-mercaptoethanol, 10% glycerol, and 2 mg/ml BSA. At the indicated time intervals, aliquots were withdrawn for the determination of enzymatic activities toward ^{32}P-Ser-phosphorylase a (Phlase a) (in the presence of 2 mM EDTA, □), ^{32}P-Tyr-IgG (in the presence of 20 mM $MgCl_2$, ○), and PNPP (in the presence of 20 mM $MgCl_2$, X), as described in "Experimental Procedures" 3.5 and 3.5.3

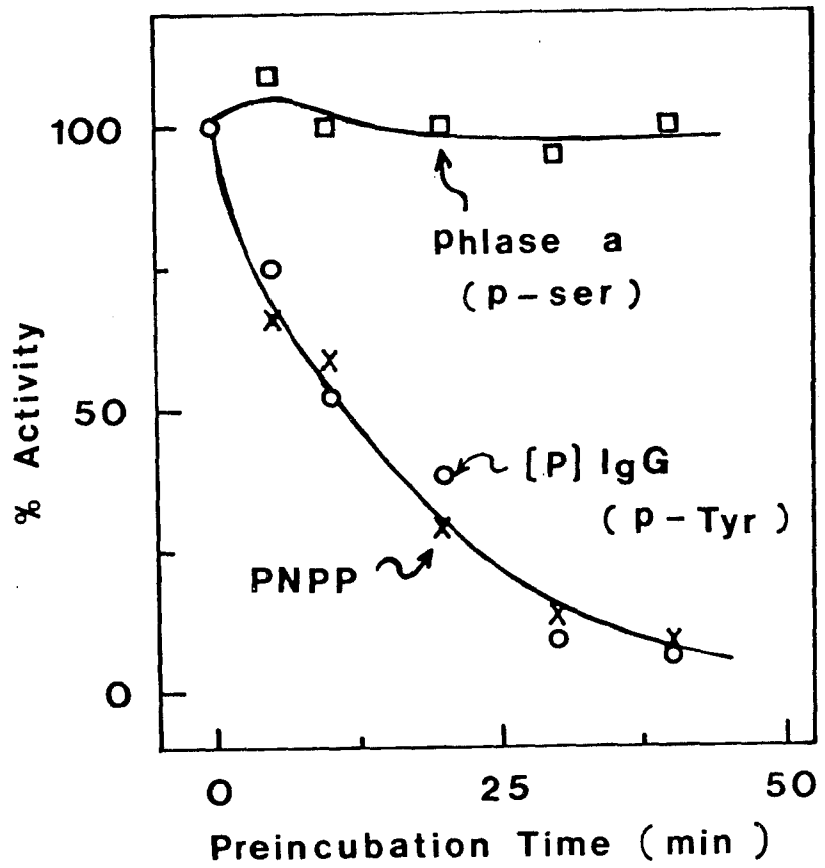


Fig. 11. Effects of P_i concentration on phosphatase activities toward ^{32}P-Ser-phosphorylase a, ^{32}P-Tyr-IgG, and PNPP. The enzymatic activities were measured as described in "Experimental Procedures" 3.5 and 3.5.3, in the presence of the indicated amounts of P_i. Enzymatic activity is given as a percentage of the activity in the absence of P_i (^{32}P-Ser-phosphorylase, □, ^{32}P-Tyr-IgG, O; PNPP, X). Phlase a, phosphorylase a

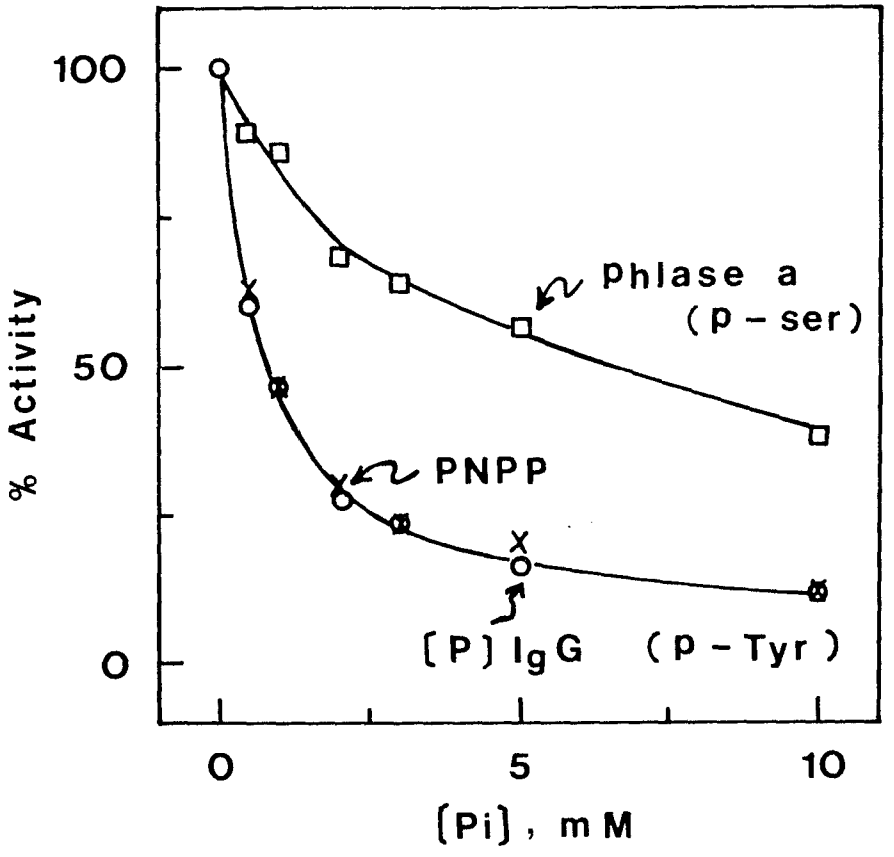
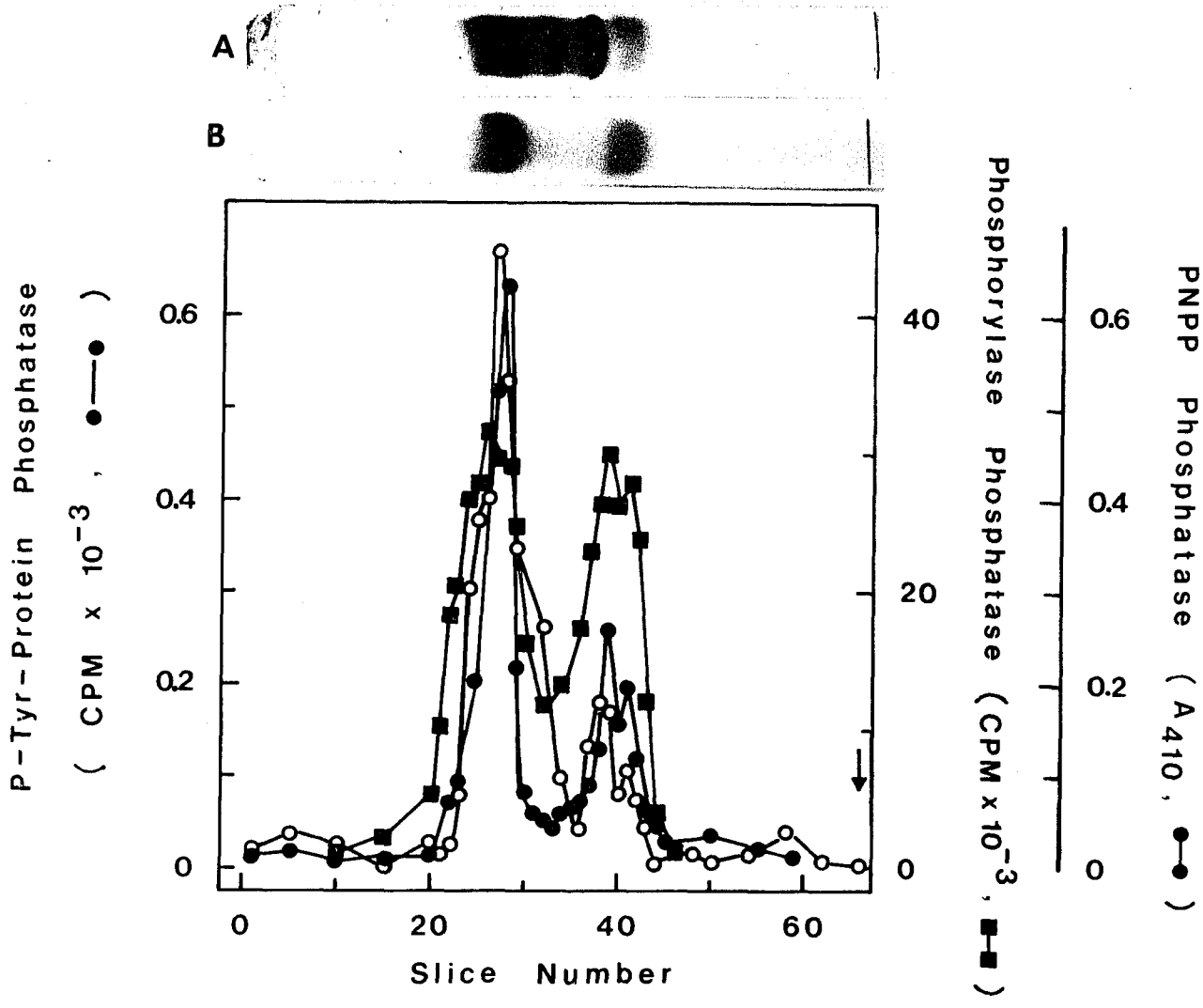


Fig. 12. Polyacrylamide gel electrophoresis of protein phosphatase-3C. Native gel electrophoresis was carried out as follows. 10 µg of enzyme was loaded onto each of three 7% polyacrylamide gels. Following electrophoresis, one was stained for protein (A), a second was stained for alkaline phosphatase (B), and a third was sliced and assayed for ^{32}P-Tyr-IgG phosphatase activity as described in "Experimental Procedures" 3.5 and 3.5.3. The dye front is indicated by the wires and the arrow.



4.2.4 Protein Phosphatase-4

Substrate Specificity -- The relative activities of the type 4 phosphatase toward a variety of (P)Tyr-, (P)Ser-, and (P)Thr-proteins is presented in Table 11. Of the substrates tested, the enzyme is most active toward ^{32}P-Ser-casein, in agreement with previous observations concerning the substrate specificity of the enzyme (151). Phosphatase activity toward the two (P)Tyr-proteins is greater at pH 8.6 than at pH 7.0, in contrast to the results obtained with ^{32}P-Ser-casein and ^{32}P-Ser-glycogen sythase. Like the two (P)Tyr-proteins, ^{32}P-Thr-inhibitor-1 and ^{32}P-Ser-phosphorylase a are dephosphorylated at a higher rate when assayed at alkaline rather than at neutral pH. ^{32}P-Tyr-IgG was dephosphorylated at a rate roughly 5 times that of ^{32}P-Tyr-casein. These values, however, represent only about 2 and 0.4%, respectively, of the activity toward ^{32}P-Ser-casein, when enzymatic activities are measured at pH 8.6.

Effects of Enzyme and Substrate Concentratiion -- Fig. 13 shows that, in the presence of Mg^{2+}, the reaction rate is linearly proportional to the increase of type 4 phosphatase concentration. When the concentration of ^{32}P-Tyr-IgG was increased from 0.25 to 0.5 nM, the reaction rate doubled at all enzyme concentrations examined. The data indicate that

the concentration of ^{32}P-Tyr-IgG (0.25 nM) used in most of the present studies is much lower than the K_m value of the enzyme for this phosphoprotein.

Effects of Mg^{2+} and Mn^{2+} -- As shown in Fig. 14, the enzyme is almost completely inactive in the absence of divalent cation. Both Mg^{2+} and Mn^{2+} activated the dephosphorylation in a concentration dependent manner. The Mg^{2+} activation reaction follows Michaelis-Menten kinetics with an apparent K_m of about 6 mM. On the other hand, the Mn^{2+} activation reached a maximum at about 2 mM, with higher concentrations of Mn^{2+} causing inhibition. At optimal concentrations, both Mg^{2+} and Mn^{2+} activated the enzymatic activity to about the same extent. At a concentration of 5 mM, Co^{2+}, Ca^{2+}, and Zn^{2+} were not effective. When Mg^{2+} (10 mM) was used as an activator, additions of 50 μM Zn^{2+} abolished the enzymatic activity. Additions of 5 mM KP_i, $NaPP_i$, ATP, or NaF results in an inhibition of the enzymatic activity 80, 95, 80, and 90% respectively.

Comigration of (P)Ser- and (P)Tyr-Protein Phosphatase Activities on Polyacrylamide Gel Electrophoresis -- To ensure that the observed (P)Tyr-protein phosphatase activity is catalyzed by the M_r 49,000 phosphatase-4 and not by a contaminating activity, native polyacrylamide gel electrophoresis of this enzyme was carried out. The gel was

cut into 1 mM slices, eluted into buffer, and assayed for ^{32}P-Ser-casein and ^{32}P-Tyr-IgG phosphatase activity. The results indicate that the ^{32}P-Ser-casein and ^{32}P-Tyr-IgG phosphatase activities comigrate (not shown). Therefore, it is unlikely that the (P)Tyr-protein phosphatase activity associated with the type 4 enzyme is due to a contaminating enzyme.

Table 11. Dephosphorylation of ^{32}P-Ser-, ^{32}P-Thr-, and ^{32}P-Tyr-proteins by phosphatase-4. Reactions were carried as described in "Experimental Procedures" (3.5) for 10 min in the presence of $MgCl_2$ (10 mM) at pH 7.0 and 8.6, and ^{32}P-protein (0.8 nM).

Substrate	Activity (fmol/min/ml)	
	pH	
	7.0	8.6
^{32}P-Ser-phosphorylase	304	390
^{32}P-Ser-casein	4965	3702
^{32}P-Ser-glycogen synthase	259	228
^{32}P-Thr-inhibitor-1	46	80
^{32}P-Tyr-IgG	51	67
^{32}P-Tyr-casein	9	15

Fig. 13 Effects of protein phosphatase-4 concentrations on the dephosphorylation of ^{32}P-Tyr-IgG. The reaction was carried out at pH 7.0 in the standard assay mixture (Experimental Procedures 3.5) containing 10 mM $MgCl_2$, 0.25 nM (○-○), or 0.5 nM (●-●) ^{32}P-Tyr-IgG, and various concentrations of phosphatase as indicated

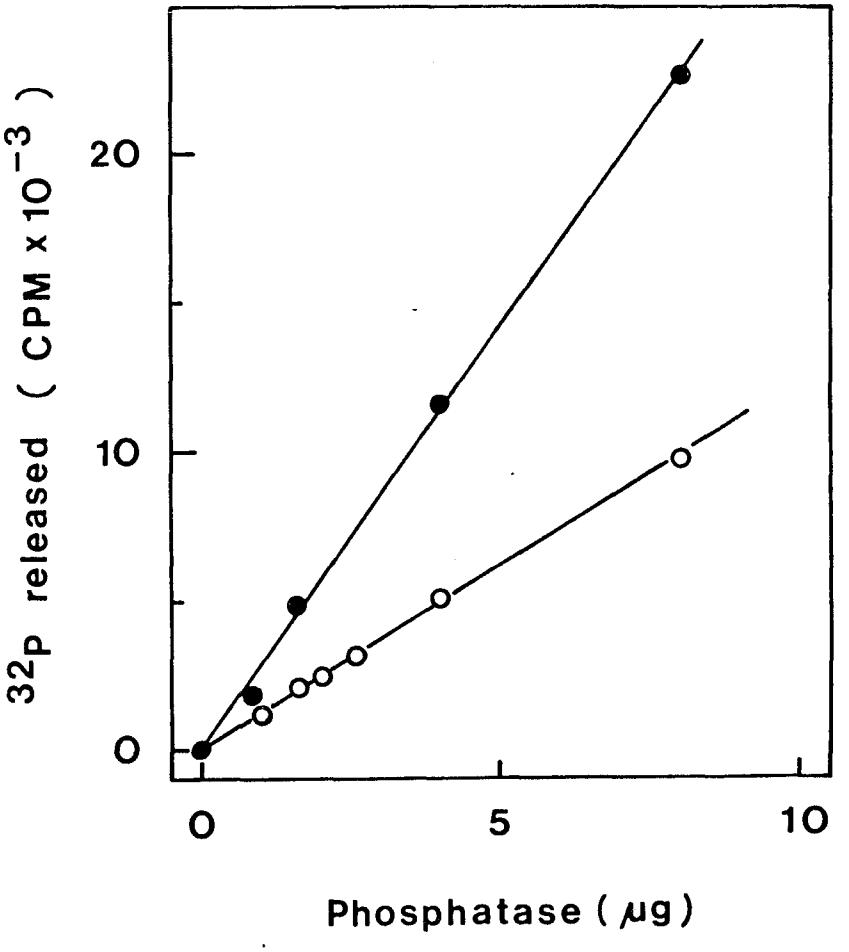
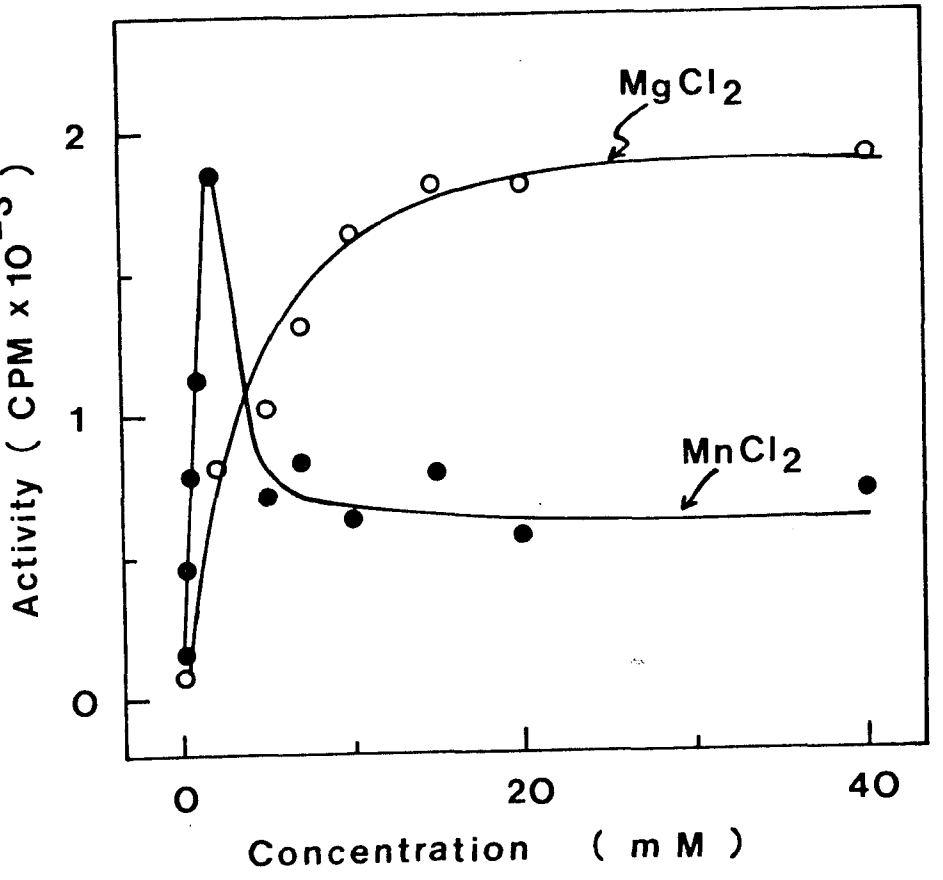


Fig. 14 Effects of $MgCl_2$ and $MnCl_2$ concentrations on the activity of protein phosphatase-4 toward ^{32}P-Tyr-IgG. The reaction was carried out at pH 7.0 in the standard reaction mixture (Experimental Procedures 3.5) containing 0.25 nM ^{32}P-Tyr-IgG, 1.6 μg of phosphatase and various concentrations of $MgCl_2$ (○-○) or $MnCl_2$ (●-●) as indicated



4.3 Phosphotyrosyl-Protein Phosphatases in Animal Tissue Extracts

Table 12 shows a comparison of phosphatase activity in a bovine heart extract toward a representative (P)Tyr- and (P)Ser-protein. [1] Under their respective optimal conditions for dephosphorylation, the total phosphatase activity toward ^{32}P-Ser-casein is slightly greater than toward ^{32}P-Tyr-IgG. However, two striking differences are apparent. Firstly, the ^{32}P-Ser-casein phosphatase activity is much greater at neutral than at other pH values, while the ^{32}P-Tyr-IgG phosphatase activity is much greater at acidic than neutral values. Secondly, the ^{32}P-Ser-casein phosphatase activity is activated by Mn^{2} and Mg^{2+}, whereas that toward ^{32}P-Tyr-IgG is inhibited by divalent cation. These differences suggest that either distinct enzymes are active against these two species of phosphoprotein or that the substrates themselves, by virtue of their structure or phosphoamino acid content, dictate different catalytic requirements for dephosphorylation.

In order to further investigate these issues, the bovine heart extract was applied to a DEAE-cellulose column

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1. Assays performed using molybdate extraction of $^{32}P_i$ reveal that the dephosphorylation of these substrates is not due to proteolytic activity.

equilibrated in a pH 7.0 buffer. The column was then washed with 3 volumes of equilibration buffer, followed by 3 volumes of the same buffer plus 0.5 M KCl. The two DEAE-bound and -unbound fractions were then tested for phosphatase activity (Table 13). The unbound fraction contains a ^{32}P-Tyr-IgG phosphatase activity which is higher at pH 5.0 than 7.2, while the bound fraction displays the opposite characteristic. Also, the bound fraction is stimulated by EDTA, indicating a high degree of sensitivity to inhibition by divalent cation. Similar experiments performed with ^{32}P-Ser-casein reveal trace activity in the unbound fraction and divalent cation-stimulated, neutral-optimal activity in the bound fraction (not shown). It is apparent from these data that at least two species of ^{32}P-Tyr-IgG phosphatase exist, one with an acidic pH optimum and another with a neutral optimum which is stimulated by EDTA. In addition, the first of these species must be distinct from protein phosphatases-1-4, as the latter enzymes bind to DEAE-cellulose under the conditions employed.

Table 12. Comparison of ^{32}P-Tyr-IgG and ^{32}P-Ser-casein phosphatase activity in crude extract of bovine heart 10 g of bovine heart was homogenized as described in "Experimental Procedures" (3.3.3) and assayed for ^{32}P-protein (0.4 nM) phosphatase activity at the specified pH in the presence of no effector, EDTA (1 mM), $MnCl_2$ (0.5 mM), or $MgCl_2$ (20 mM). The reaction was initiated by the addition of enzyme and incubated for 20 min.

Substrate	^{32}P-Tyr-IgG			^{32}P-Ser-casein		
pH	5.0	7.2	8.6	5.0	7.2	8.6
	Activity (pmol/min/g tissue)					
control	259	81	67	62	53	82
EDTA	205	112	69	77	35	19
Mn^{2+}	147	56	56	111	338	89
Mg^{2+}	155	54	55	87	307	121

Table 13. Properties ^{32}P-Tyr-IgG phosphatase activity in DEAE-cellulose-bound and -unbound fractions of bovine heart extract An extract from 10 g bovine heart was applied to 5 ml DE-52 (Whatman) and washed with 15 ml Buffer C. The resin was then washed with 15 ml Buffer C plus 0.5 M KCl. The breakthrough and 0.5 M KCl eluate fractions were assayed for ^{32}P-Tyr-IgG (0.4 nM) phosphatase activity at the specified pH in the presence of no effector, EDTA (1 mM), or $MnCl_2$ (0.5 mM), and enzyme for 20 min.

Fraction	Unbound		Bound	
pH	5.0	7.2	5.0	7.2
	Activity (pmol/min/g tissue)			
control	195	51	18	54
EDTA	207	33	33	96
Mn^{2+}	132	54	9	12

4.3.1 Tissue Distribution

Several other tissues were examined to determine whether the results obtained with bovine heart are unique to this tissue. Extracts of bovine brain, rabbit liver, and human kidney were applied to DEAE-cellulose and processed identically to the heart extract. The crude extracts, DEAE-bound and -unbound fractions were then assayed for ^{32}P-Tyr-IgG activity at pH 5.0 and 7.2 (in the presence of EDTA). Table 14 shows that, as in bovine heart, bovine brain and human kidney contain two types of ^{32}P-Tyr-IgG phosphatase activity: (a) one which does not bind to DEAE-cellulose which is greater at acidic than at neutral pH, and (b) one which binds to this resin which is greater at neutral than at acidic pH. Somewhat different results are obtained with rabbit liver, which contains an acid-optimal, DEAE-bound activity. These data indicate that the enzymatic species present in bovine heart are similar to those present in certain other mammalian tissues, but that additional species of (P)Tyr-protein phosphatase may be present in liver.

Rabbit liver contains the most activity per g of tissue, followed by human kidney, bovine brain, and bovine heart. In rabbit liver, the sum of activities in the two DEAE fractions is much greater than in the crude extract. This

finding may be explained by activation of phosphatase(s) during the chromatographic procedure. Such activation might occur by limited proteolysis of the phosphatase(s) or by the separation of inhibitor molecules.

Table 14. Tissue distribution of ^{32}P-Tyr-IgG phosphatase activity 10 g each of bovine heart, bovine brain, human kidney, and rabbit liver were homogenized as described (Experimental Procedures 3.3.3), except that no $(NH_4)_2SO_4$ fractionation was performed. The extracts were then applied to 10 ml DE-52 and processed as described in the legend to Table 13. ^{32}P-Tyr-casein phosphatase activities in the crude extracts, DEAE-bound and -unbound fractions were assayed for 20 min at pH 5.0 and 7.2 in the presence of EDTA (1 mM) using ^{32}P-Tyr-IgG phosphatase (0.4 nM)

		Activity (pmol/min/g tissue)		
tissue	pH	crude extract	unbound	bound
heart	5.0	324	191	12
	7.2	125	54	93
brain	5.0	325	200	50
	7.2	153	39	154
kidney	5.0	573	316	42
	7.2	153	27	195
liver	5.0	886	1074	468
	7.2	540	584	392

4.3.2 Subcellular Distribution

The subcellular distribution of ^{32}P-Tyr-IgG phosphatase activity in bovine cerebellum was examined (Table 15). Fractions representing nuclei and plasma membranes (P1), mitochondria and plasma membranes (P2), microsomes (P3) and cell sap (S) were assayed for phosphatase activity at pH 7.0 in the presence of EDTA. The results show that, under the conditions examined, this tissue contains predominantly soluble ^{32}P-Tyr-IgG phosphatase activity. The P1 fraction also contains appreciable ^{32}P-Tyr-IgG phosphatase activity. Activity at pH 5.0 was not measured. These results indicate that the majority of ^{32}P-Tyr-IgG phosphatase activity is cytosolic, but that significant levels of this activity are also present in other subcellular locations. Because the levels of this activity are higher in the cytosolic fraction and because the author desired to compare this activity with the (soluble) classic protein phosphatases, no further experiments were performed with the particulate enzyme(s).

Table 15. Subcellular distribution of ^{32}P-Tyr-IgG phosphatase activity in bovine cerebellar cortex Bovine cerebellum was homogenized and fractionated as described in "Experimental Procedures" 3.8. Fractions were assayed for ^{32}P-Tyr-IgG (0.6 nM) phosphatase activity for 20 min at pH 7.2 in the presence of EDTA (1 mM) and initiated by the addition of enzyme as described in "Experimental Procedures" 3.5. All measurements are the averages of two fractionations. RSA (Relative specific activity) is defined as the ratio of % recovered activity/% recovered protein.

Fraction	Protein	(P)Tyr-Protein Phosphatase		Lactate Dehydrogenase	Succinate Dehydrogenase
	mg/g tissue	Units[a]	RSA	RSA	RSA
P1 Nuclei	38	334	0.8	0.4	1.1
P2 Mitochondria	27	155	0.5	0.4	1.9
P3 Microsomes	7.5	39	0.4	0.3	0
S Cell Sap	22.5	575	2.2	3.2	0
Total	95	1103			
Homogenate	92	813			
Recovery (%)	103	136		100	82

[a] Units are expressed as fmol/min.

4.3.3 Separation of Bovine Heart Phosphotyrosyl-Protein Phosphatases by DEAE-cellulose Chromatography

As the aforementioned data clearly indicate that bovine heart contains more than one species of ^{32}P-Tyr-IgG phosphatase, the elution profile of this activity from DEAE-cellulose was examined in detail. After applying the crude extract to this resin and washing to remove unbound protein, the column was developed with a linear 0.05 to 0.5 M KCl gradient. Using ^{32}P-Tyr-IgG as substrate, fractions were assayed for (P)Tyr-protein phosphatase activity under acidic and neutral conditions, in the presence of the indicated effectors (Fig. 15a, 15c). The data indicate that two major and one minor active peaks of (P)Tyr-protein phosphatase are detected under the assay conditions examined. These are termed Y-1, Y-2, and Y-3, and are eluted at 0.05, 0.12-0.21, and 0.27-0.31 M KCl, respectively. The greatest activity is observed at pH 5.0 in the DEAE-cellulose breakthrough (Y-1) fractions (Fig. 15a). The Y-2 and -3 phosphatase peaks are more active at pH 7.2 than 5.0, and are stimulated by EDTA (Fig. 15c). Mn^{2+} and Mg^{2+} are inhibitory. When measured at pH 5.0 in the absence of added effectors, peaks Y-1, -2, and -3, represent about 80, 15, and 5% of the total ^{32}P-Tyr-IgG phosphatase activity recovered from the resin. When measured at pH 7.2 in the presence of EDTA, these levels are about 33, 55, and

12%.

Fig. 15d also shows the elution positions of protein phosphatase-1-4: phosphatase-1, measured as an ATP/Mg^{2+}-F_A-activated ^{32}P-Ser-phosphorylase phosphatase (■-■); phosphatase-2 (calcineurin), measured as a Ca^{2+}/calmodulin-activated ^{32}P-Thr-inhibitor-1 phosphatase (elution profile not shown); phosphatases-3A, -3B, and -3C, measured as Mn^{2+}-stimulated ^{32}P-Ser-phosphorylase phosphatase (□-□); and phosphatase-4 measured as Mg^{2+}-activated ^{32}P-Ser-casein phosphatase (elution profile not shown). The elution profiles of acid (▲-▲) and alkaline (△-△) phosphatase activity toward PNPP are shown in Fig. 15b. The data indicate that phosphatase Y-1, like the bovine heart acid phosphatase activity, does not bind to DEAE-cellulose under the conditions employed. Consequently, this activity must differ from all four forms of classical protein phosphatases. Phosphatase Y-2 elutes from the ion-exchange resin at a similar salt concentration as protein phosphatase-1, but, unlike this enzyme, is stimulated by EDTA. In addition, purified phosphatase-1 has little (P)Tyr-protein phosphatase activity (Results 4.2.1).

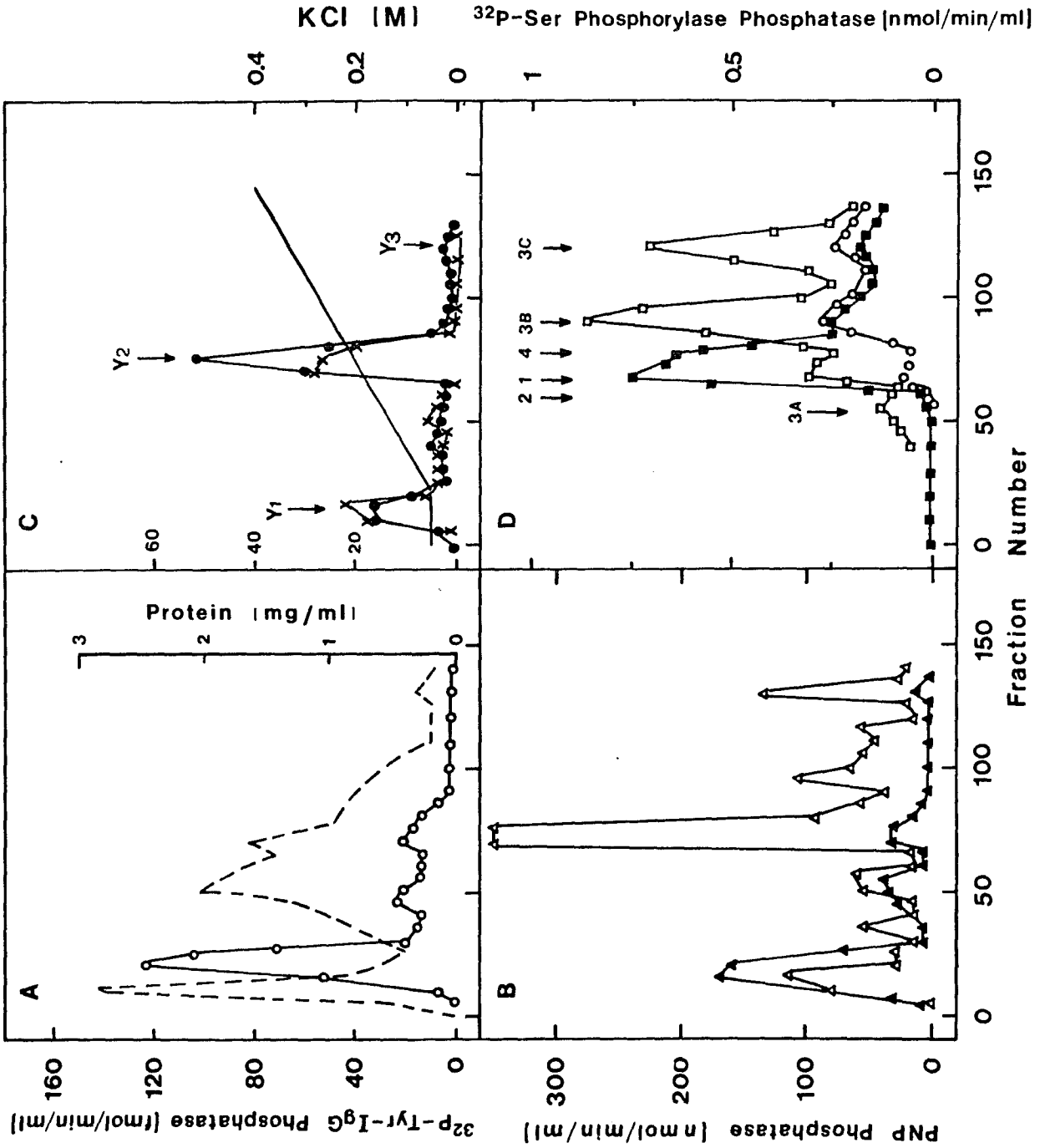

KCl (M)
32P-Ser Phosphorylase Phosphatase (nmol/min/ml)
Protein (mg/ml)
32P-Tyr-IgG Phosphatase (fmol/min/ml)
PNP Phosphatase (nmol/min/ml)
Fraction Number
A
B
C
D
Y1
Y2
Y3
3A
3B
3C
2 1 4

Fig. 15 Separation of phosphatases by DEAE-cellulose chromatography. The 55% $(NH_4)_2SO_4$ fraction of bovine cardiac extract was chromatographed on a DEAE-cellulose column as described in "Experimental Procedures" 3.3.3. (^{32}P)Tyr-protein phosphatase activity was measured with 1.0 nM ^{32}P-Tyr-IgG at pH 5.0 (A, ○ - ○), or pH 7.0 in the presence of 1 mM EDTA (C, ● - ●), or 10 mM $MgCl_2$ (C, X - X). Phosphorylase phosphatase activity was measured with 10 μM ^{32}P-Ser-phosphorylase a following preincubation for 20 min in the presence (D, ■ - ■) and absence (D ○ - ○) of 1 mM $MgCl_2$, 0.5 mM ATP, and F_A. Phosphorylase phosphatase activity was also measured in the presence of 0.5 mM $MnCl_2$ (D, □ - □). Acid (B, ▲ - ▲) and alkaline (△ - △) phosphatase activities were determined as described (Experimental Procedures 3.5.3). Protein (A, ---) was measured by the procedure of Bradford (190)

4.3.4 Purification of Phosphotyrosyl-Protein Phosphatase Y-1 from Bovine Heart

Phosphatase Y-1, which represents the major ^{32}P-Tyr-IgG phosphatase activity (at pH 5.0) in crude extracts of the tissues examined, was purified as follows. The 55% $(NH_4)_2SO_4$ fraction of a bovine heart extract was applied to a DEAE-cellulose column and the phosphatase Y-1, as well as the other unabsorbed proteins, were washed from the column with 0.05 M KCl. The enzyme was then chromatographed on Sephadex G-75, followed by SP-sephadex and Matrex Red A. The enzyme appeared as two peaks upon gel filtration (Fig. 16a); a minor species of M_r approximately 40,000 and a major species of M_r approximately 13,000. This smaller species coeluted with acid phosphatase activity. These two activities bound tightly to the cation-exchange resin, SP-sephadex, eluting at about 0.8 M KCl (Fig. 16b). Finally, the (P)Tyr-protein and acid phosphatase activities were purified from the bulk of the remaining proteins by chromatography on Matrex Red A. The enzymatic activities eluted at about 5% glycerol (Fig. 16c). Polyacrylamide gel electrophoresis indicated that the enzyme preparation contained several protein bands. The major protein band comigrated with both (P)Tyr-protein and acid phosphatase activity (Fig. 17), suggesting that these two activities are contained in the same molecule. A summary of the

purification of the enzyme is provided in Table 16. The phosphatase was purified about 350-fold, with a yield of 5%. The ratio of (P)Tyr-protein to acid phosphatase activity remains essentially constant after the first purification step. The purified enzyme is stable upon storage at -20°C in 50% glycerol.

pH Optimum -- In order to study various biochemical properties of the (P)Tyr-protein phosphatase activity of the Y-1 enzyme, ^{32}P-Tyr-casein phosphorylated by the EGF receptor/kinase was employed as substrate. This substrate could be phosphorylated to a much greater extent than ^{32}P-Tyr-IgG and was thus essential for the determination of kinetic data as well as for comparisons of (P)Tyr-protein, (P)Ser/Thr-protein, and PNP phosphatase activities. Both the ^{32}P-Tyr-casein and the PNP phosphatase activates display optima for activity at acidic pH values (not shown). Similar results are obtained when ^{32}P-Tyr-IgG is used as substrate. These findings are similar to those reported for human prostate acid phosphatase (Results 4.1.1), but differ from those for an acid phosphatase from human astrocytoma cells (158).

Kinetics of Dephosphorylation -- The enzymatic activity toward both PNPP and ^{32}P-Tyr-casein follow Michaelis-Menten kinetics (not shown). The K_m and V_{max} are 67 μM and 169

μmol/min/mg for the PNP phosphatase activity, and 1.0 μM and 67 nmol/min/mg for the ^{32}P-Tyr-casein phosphatase activity.

Effect of Inhibitors -- The enzymatic activity was examined in the presence of compounds which have been reported to inhibit various acid and (P)Tyr-protein phosphatases (Table 17). Both the PNP and ^{32}P-Tyr-casein phosphatase activities were determined with the substrates present at their respective K_m values. L(+)-tartrate, which is a potent inhibitor of the PNP phosphatase activity of human prostatic acid phosphatase, as well as the (P)Tyr-protein phosphatase activity associated with this enzyme (Results 4.1.1), is ineffective as an inhibitor of the Y-1 enzyme toward either substrate. Vanadate and molybdate are both inhibitory at low concentrations. Moreover, the degree of inhibition of either activity is similar. NaP_i is also an effective inhibitor of the enzyme, while NaF, Zn^{2+}, and inhibitors-1 and -2 are ineffective at the concentrations examined.

Substrate Specificity -- The activities of phosphatase Y-1 and human placental alkaline phosphatase were measured toward an assortment of (P)Ser-, (P)Thr-, and (P)Tyr-proteins (Table 18). The activity of phosphatase Y-1 toward the two (P)Tyr-proteins is much greater than toward proteins labeled at serine or threonine residues. In

addition, this phosphatase is more specific for (P)Tyr-proteins than placental alkaline phosphatase. The ratio of ^{32}P-Tyr- to ^{32}P-Ser-casein phosphatase activity is about 183 for the Y-1 enzyme and 14 for the alkaline phosphatase. These data indicate that the phosphatase Y-1 is extremely specific for (P)Tyr-proteins.

Table 16. Co-purification of ^{32}P-Tyr-IgG and acid phosphatase activities Phosphatase Y-1 was purified from 200 g bovine heart as described in "Experimental Procedures" (3.3.3.1). Phosphatase activities were determined at pH 5.0 using ^{32}P-Tyr-IgG (1.2 nM) and PNPP (4 mM).

[a] Units are expressed as fmol/min for ^{32}P-Tyr-IgG and umol/min for PNP phosphatase activities.

Fraction	Total protein	Specific activity on		
		PNPP	^{32}P-Tyr IgG	(A/B)
	mg	units/mg[a]		
1. Crude Extract	740	93	136	(0.68)
2. Ammonium sulfate fractionation	492	125	173	(0.72)
3. DEAE-cellulose	343	174	218	(0.79)
4. Sephadex G-75	30	500	573	(0.76)
5. SP-Sephadex	6.7	1373	1791	(0.77)
6. Red A	0.02	34309	47491	(0.72)

Table 17. Effects of various compounds on (P)Tyr-protein and acid phosphatase activities The phosphatase activities were measured at pH 5.0 with ^{32}P-Tyr-casein (1.2 μM) or PNPP (4 mM) in the absence or presence of effectors as indicated.

Addition	Activity on	
	^{32}P-Tyr-casein	PNPP
	%	
None	100	100
Molybdate, 10 μM	14	26
100 μM	1	2
Vanadate, 10 μM	67	75
100 μM	27	50
L(+)-Tartrate, 5 mM	82	90
NaF, 5 mM	83	95
KP_i, 5 mM	28	54
$ZnCl_2$, 100 μM	80	90
Inhibitor-1, 10 nM	99	102
Inhibitor-2, 30 nM	90	115

Table 18. Substrate specificity of phosphatase Y-1
Enzymatic activity was measured at pH 5.0 (phosphatase Y-1) or 8.6 (human placental alkaline phosphatase) as described in "Experimental Procedures" (3.5). Substrate concentrations were as follows: ^{32}P-Ser-casein and ^{32}P-Thr-inhibitor-1, 1 μM; ^{32}P-Ser-phosphorylase, 8 μM; ^{32}P-Tyr-casein, 1.3 μM; ^{32}P-Tyr-myosin light chain (M.L.C.), 0.7 μM. Activity is expressed as nmol/min/mg.

Substrate	Phosphatase Y-1	Human Placental Alkaline Phosphatase
^{32}P-Ser-phosphorylase	1.9	1.5
^{32}P-Ser-casein	0.3	3.5
^{32}P-Thr-inhibitor-1	0	12
^{32}P-Tyr-casein	55	48
^{32}P-Tyr-M.L.C	10	13

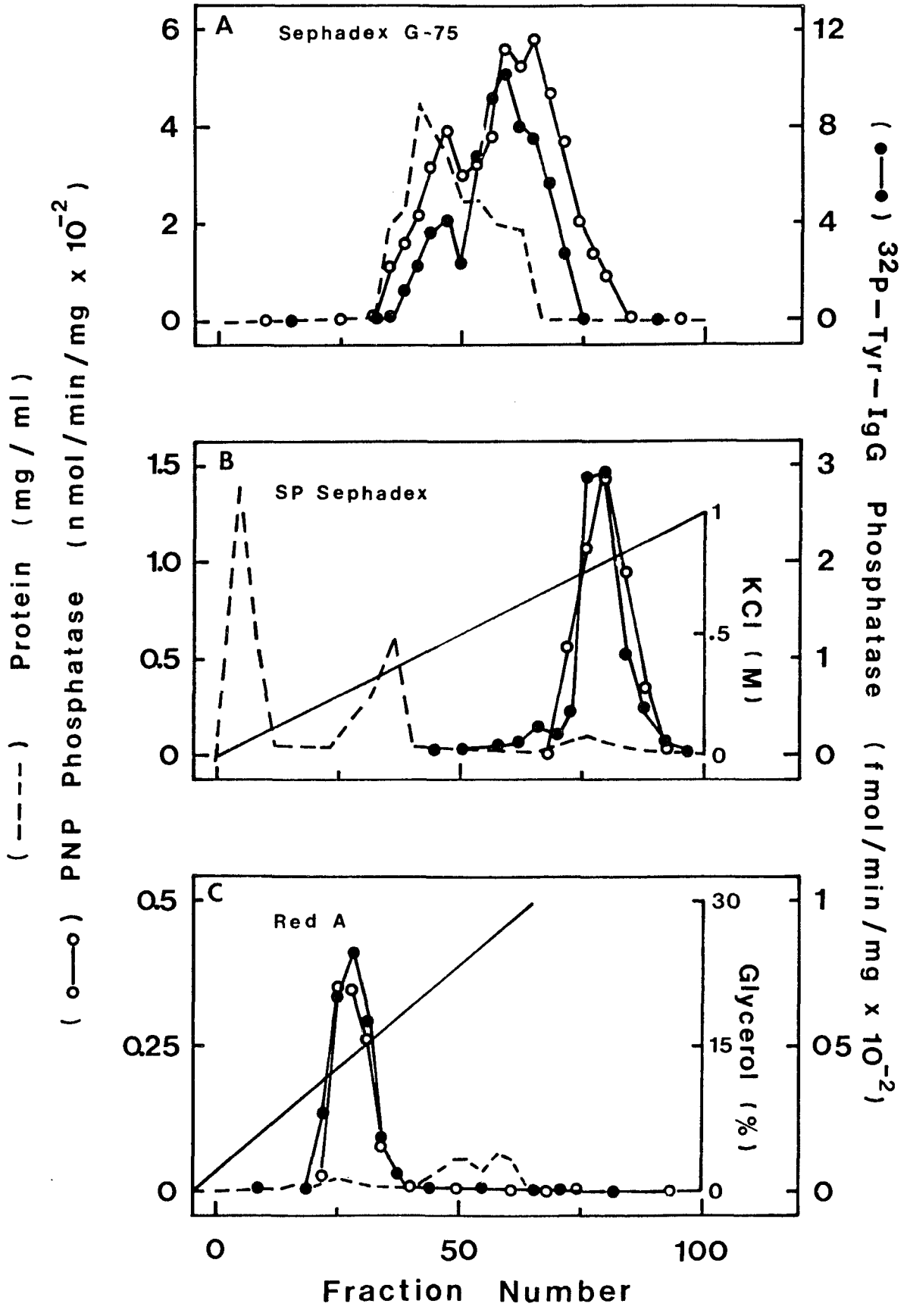
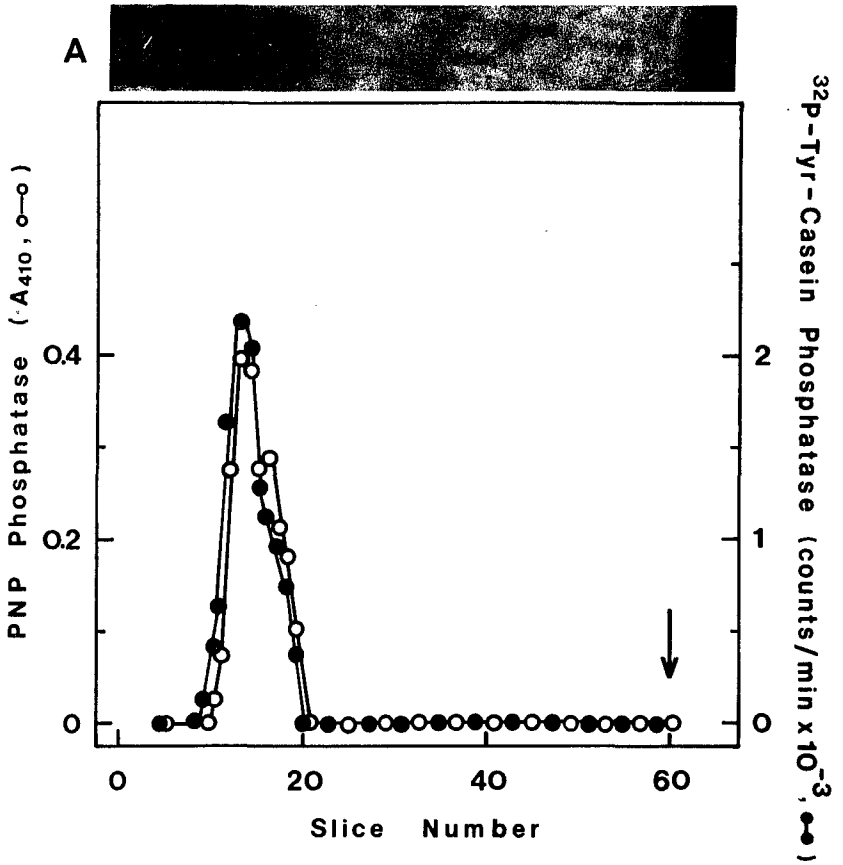

A
Sephadex G-75
B
SP Sephadex
C
Red A
KCl (M)
Glycerol (%)
Fraction Number
(----) Protein (mg/ml)
(o—o) PNP Phosphatase (nmol/min/mg x 10^{-2})
(●—●) ^{32}P-Tyr-IgG Phosphatase (fmol/min/mg x 10^{-2})

Fig. 16 Isolation of (P)Tyr-protein phosphatase Y-1. An extract of bovine heart (200 g) was chromatographed on DEAE-cellulose, and the breakthrough fraction was applied to a Sephadex G-75 column, followed by chromatography on SP-sephadex and Matrex Red A as described (Experimental Procedures 3.3.3.1). Fractions were assayed for acid phosphatase at pH 5.0 (O - O) and for ^{32}P-Tyr-IgG phosphatase (0.6 nM) activity at pH 5.0 (● - ●), as described (Experimental Procedures 3.5 and 3.5.3). Protein (---) was measured by the method of Bradford (190)

Fig. 17 Co-migration of (P)Tyr-protein and acid phosphatase activities on polyacylamide gel electrophoresis. 0.5 µg of enzyme was loaded onto each of two 7% polyacrylamide gels. Following electrophoresis in a pH 7.0 buffer system (183), one gel was silver-stained for protein (A), and the second was sliced and assayed for ^{32}P-Tyr-IgG and acid phosphatase activities as described in "Experimental Procedures" 3.5 and 3.5.3



4.3.5 Purification of Phosphotyrosyl-Protein Phosphatase Y-2 from Bovine Heart

Since peak Y-2 (Fig. 15c) appears to be the major neutral ^{32}P-Tyr-protein phosphatase activity in cardiac muscle and brain, further purification of this activity from bovine heart was carried out as follows: The 55% $(NH_4)_2SO_4$ fraction was applied to a DEAE-cellulose column and phosphatase Y-1 together with the bulk of unabsorbed proteins was eluted out of the resin with 0.05 M KCl. The column was then developed with 0.12 M KCl. Phosphatase Y-2 and part of protein phosphatase-1 activity were eluted out of the column by this ionic strength (Fig. 18a). The enzyme was rechromatographed on a second DEAE-cellulose column (Fig. 18b) followed by chromatography on Sephacryl S-200 and polylysine-Sepharose 4B. As shown in Fig. 18c, phosphatase Y-2 migrates on Sephacryl S-200 as a single peak coeluting with alkaline phosphatase activity. This alkaline phosphatase activity, however, separates from the phosphatase Y-2 activity on polylysine-Sepharose (Fig. 18d). A summary of the purification procedure is presented in Table 19. The data indicate that phosphatase Y-2 was purified about 25-fold with a yield of 2%. The observed low yield and degree of purification may partially be due to the fact that the enzymatic activity becomes very unstable after the second DEAE-cellulose chromatography step. The stability of Y-2

can be increased in the presence of 50% glycerol. The enzyme has a half-life of about 2 months when stored at -20°C in a buffer solution containing 20 mM Tris-HCl, pH 7.0, 10 mM 2-mercaptoethanol, and 50% glycerol. Polyacrylamide gel electrophoresis of the phosphatase Y-2 preparation reveals multiple protein bands indicating that it is not homogeneous (not shown).

Molecular Weight -- The $s_{20,w}$ and Stokes' radius of (P)Tyr-protein phosphatase Y-2 were estimated to be 4.1 and 3.8 nm, respectively. Using the $s_{20,w}$ and Stokes' radius values obtained and assuming a partial specific volume of 0.725 ml/g, the molecular weight of the enzyme was calculated (194) to be 65,000.

Stimulation by Chelating Agents -- The activity of phosphatase Y-2 toward ^{32}P-Tyr-IgG is stimulated by EDTA in a concentration dependent manner (Fig. 19). At saturating concentrations of EDTA, the enzymatic activity increases about 4- to 5-fold. Half-maximal stimulation of the activity occurs at approximately 15 μM EDTA. The enzymatic activity toward ^{32}P-Tyr-casein is also greatly stimulated by EDTA (Table 22), suggesting that the EDTA exerts its action on the phosphatase rather than on the phosphoprotein substrate. The effects of several other chelating agents on the enzymatic activity have been examined.

Hydroxylquinoline and desferrioxamine are as effective, while phenanthroline and EGTA are less effective than EDTA in stimulating the enzymatic activity (Table 20). On the other hand, succinic acid is slightly inhibitory. The data indicate that the stimulatory effect of EDTA may be attributed to its ability to chelate divalent cations.

Effects of pH -- The enzymatic activity toward ^{32}P-Tyr-IgG, measured in the presence of 1 mM EDTA, exhibits a bell-shaped pH-activity profile with an optimum between pH 6.5 and 7.0 (not shown).

Effects of Phosphatase Inhibitors -- As shown in Table 21, the phosphatase activity toward ^{32}P-Tyr-IgG is not significantly affected by the heat-stable inhibitor-1 or -2 at concentrations which inhibit more that 90% of the cardiac protein phosphatase-1 activity toward phosphorylase a. In this respect, phosphatase Y-2 is similar to the (P)Tyr-protein phosphatases from chicken brain (161). The enzymatic activity is not significantly affected by 0.4 mM PP_i and only slightly inhibited by 0.4 mM ATP. At this concentration, PP_i and ATP act as potent inhibitors for cardiac protein phosphatase-3 isozymes and their catalytic entities of M_r 35,000 (149,150). NaF, at a concentration of 50 mM, abolishes most of the activity of protein phosphatases, but only inhibits about 45% of the phosphatase

Y-2 activity. inorganic phosphate (0.4 mM) is an effective inhibitor for both the alkaline phosphatase activity associated with the cardiac protein phosphatase-3 (149) and the intestine alkaline phosphatase activity toward ^{32}P-Tyr-histones (174), but does not significantly affect phosphatase Y-2 activity. PNPP (2 mM) has been reported to inhibit the intestine alkaline phosphatase toward ^{32}P-Tyr-histones by 75% (156). It inhibits phosphatase Y-2 more than 90% at a concentration of 0.4 mM. Vanadate, at 10 μM concentration, has been reported to selectively inhibit the (P)Tyr-protein phosphatase activity of alkaline (156,157) and acid (158) phosphatases without significantly affecting classical protein phosphatases. As shown in Table 21, vanadate is a potent inhibitor of phosphatase Y-2. The data indicate that the phosphatase Y-2 is insensitive to inhibition by inhibitors of protein phosphatases. Although phosphatase Y-2 exhibits little activity toward PNPP, it is very sensitive to inhibition by PNPP and vanadate, both of which are powerful inhibitors for either alkaline or acid phosphatase activity toward (P)Tyr-proteins.

Substrate Specificity -- In addition to ^{32}P-Tyr-IgG, the activity of phosphatase Y-2 toward several other substrates phosphorylated at serine threonine, and tyrosine, was examined under a variety of conditions. As shown in table 22, EDTA (1 mM) stimulates the enzymatic activity toward the

two ^{32}P-Tyr-proteins about 7- to 8-fold, while it inhibits that toward ^{32}P-Ser-casein about 80%. In the absence of added effectors or in the presence of EDTA, the enzyme shows no detectable activity toward ^{32}P-Ser-phosphorylase *a* or ^{32}P-Thr-inhibitor-1. The enzymatic activity toward these two substrates, however, can be elicited by 0.5 mM Mn^{2+}. This divalent cation also slightly stimulates the dephosphorylation of ^{32}P-Ser-casein as well as ^{32}P-Tyr-casein and -IgG. In all conditions examined, the rates of dephosphorylation of ^{32}P-Tyr-casein and -IgG are similar. At their respective optimal conditions, the activity toward (P)Tyr-proteins is much higher than toward (P)Ser/Thr-proteins. For example, the rate of dephosphorylation of ^{32}P-Tyr-casein measured in the presence of EDTA is about 17-fold higher than that of ^{32}P-Ser-casein measured in the presence of Mn^{2+}. The data indicate that phosphatase Y-2 is relatively specific for (P)Tyr-proteins.

Table 19. Purification of phosphatase Y-2 from bovine heart (P)Tyr-protein phosphatase Y-2 (Fig. 15) was purified from 500 g bovine heart as described in "Experimental Procedures" (3.3.3.2). Aliquots of enzyme from each purification step were assayed for ^{32}P-Tyr-(P)Tyr-protein phosphatase activity at pH 7.2 with ^{32}P-Tyr-IgG (0.6 nM) in the presence of EDTA (1 mM).

Fraction	Total protein (mg)	Specific activity (fmol/min/mg)
15,000g	8840	19
55% $(NH_4)_2SO_4$	5068	33
First DEAE-cellulose	490	149
Second DEAE-cellulose	175	144
Sephacryl S-200	17	355
Polylysine-Sepharose	6	473

Table 20. Effects of chelating agents on phosphatase Y-2 (P)Tyr-protein phosphatase activity was measured at pH 7.0 with 0.3 nM ^{32}P-Tyr-IgG as described in "Experimental Procedures" (3.5) with or without the additon of 1 mM chelating agent as indicated. Activity is expressed as the percentage of control.

Addition	Percentage activity
None	100
EDTA	435
EGTA	157
Phenanthroline	239
Desferrioxamine	400
Hydroxylquinoline	439
Succinate	74
ATP	83

Table 21. Effects of various compounds on activity of phosphatase Y-2 (P)Tyr-protein phosphatase activity was measured at pH 7.0 with ^{32}P-Tyr-IgG (0.3 nM) and EDTA (1 mM) as described in "Experimental Procedures" (3.5) with or without the addition of effectors as indicated. Activity is expressed as a percentage of control.

Addition	Percentage activity
None	100
Inhibitor-1, 40 nM	111
Inhibitor-2, 74 µg/ml	103
Pyrophosphate, 0.4 mM	118
ATP, 0.4 mM	75
NaF, 2 mM	107
NaF, 50 mM	55
P_i, 0.4 mM	124
PNPP, 0.4 mM	8
Vanadate, 10 µM	41

Table 22. Substrate specificity of phosphatase Y-2
Phosphoprotein phosphatase activity was measured at pH 7.2 as described in "Experimental Procedures" (3.5) in the absence or presence of EDTA (1 mM) or $MnCl_2$ (0.5 mM) with ^{32}P-labeled protein as indicated. Substrate concentrations ranged from 0.6 to 1.5 nM. N.D. = not determined.

Substrate	Activity (fmol/min/ml)		
	EDTA	None	$MnCl_2$
^{32}P-Ser-casein	14	60	100
^{32}P-Ser-phosphorylase	0	0	62
^{32}P-Thr-inhibitor-1	0	N.D.	523
^{32}P-Tyr-IgG	1539	208	256
^{32}P-Tyr-casein	1768	224	265

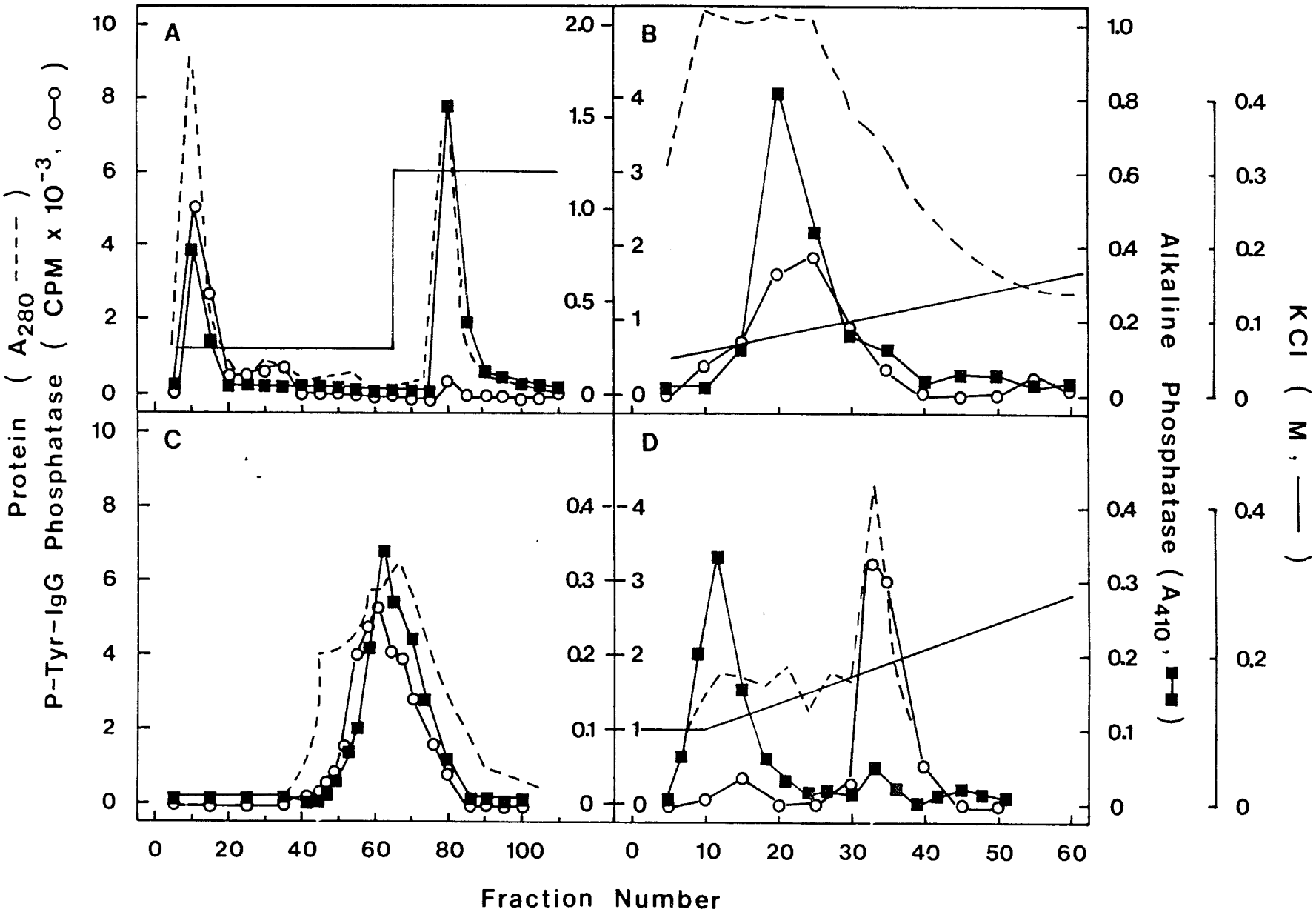
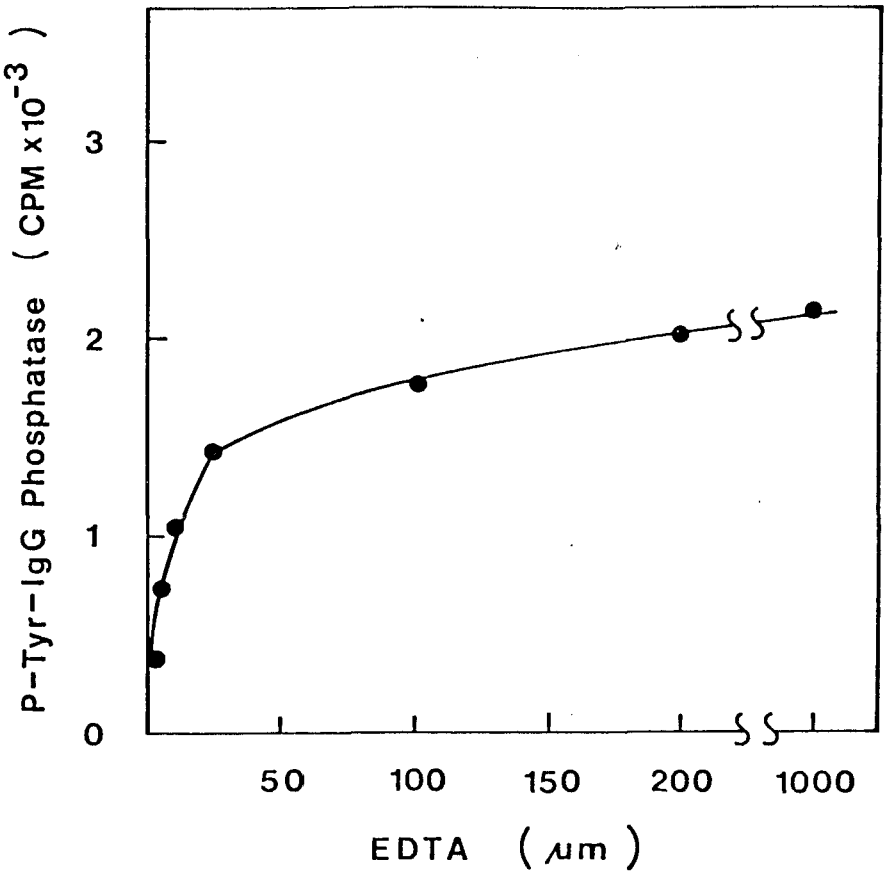

A
B
C
D
Protein (A_{280} ----)
P-Tyr-IgG Phosphatase (CPM x 10^{-3}, o—o)
Alkaline Phosphatase (A_{410}, ■—■)
KCl (M, ——)
Fraction Number

Fig. 18 Isolation of (P)Tyr-protein phosphatase Y-2. An extract of bovine heart (200 g) was twice chromatographed on DEAE-cellulose, followed by Sephacryl S-200 and polylysine-sepharose as described (Experimental Procedures 3.3.3.2). Fractions were assayed for alkaline phosphatase at pH 8.6 (■-■) and for ^{32}P-Tyr-IgG phosphatase (0.6 nM) activity at pH 7.0 in the presence of 1 mM EDTA (O - O), as described (Experimental Procedures 3.5 and 3.5.3). Protein (---) was measured by absorbance at 280 nm

Fig. 19 Effects of EDTA concentrations on (P)Tyr-protein phosphatase activity. (P)Tyr-protein phosphatase activity was measured at pH 7.0 using 0.6 nM ^{32}P-Tyr-IgG in the absence and presence of various concentrations of EDTA as indicated



DISCUSSION

One of the major problems encountered by those interested in examining (P)Tyr-protein phosphatases has been a lack of suitable physiologic substrates for study. Only a few endogenous substrates for Tyr-protein kinases are known and the effect, if any, of tyrosine phosphorylation on these proteins has not been established. In addition, endogenous substrates such as vinculin or the M_r 36,000 protein are difficult to prepare in sufficient quantities for routine assay. Experiments have been performed in which the autophosphorylated EGF or insulin receptors have been used as substrate, but, since the effects of autophosphorylation in vivo are unknown, the dephosphorylation of these proteins may have little relevance. For the most part, researchers have studied (P)Tyr-protein phosphatases using artificial substrates such as casein, histone, and derivitized phosphorylase and BSA. Not surprisingly, different enzymes are detected with these substrates. Because of the method of detection and measurement of activity, no conclusive statements can be made regarding the relative physiological importance of these enzymes.

The various enzymes which have been reported to possess relative specificity toward (P)Tyr-proteins are listed in Table 23. It is apparent from the published data that much

remains to be resolved concerning the the structure, properties, and regulation of these enzymes. At this early stage, at least two major themes have emerged: (a) PNP phosphatases, as exemplified by the acid and alkaline phosphatases, are active toward (P)Tyr-proteins; and (b) multiple forms of (P)Tyr-protein phosphatase are present in tissue extracts, and these enzymes are different than the classical protein phosphatases-1-4.

5.1 p-Nitrophenyl Phosphatases

It has become clear that all enzymes active toward small phosphomonoesters such as PNPP are likely to be active toward (P)Tyr-proteins. The converse, however, is not always true. For example, while all acid and alkaline phosphatases examined thus far are able to dephosphorylate (P)Tyr-proteins at significant rates, several (P)Tyr-proteins phosphatases have been described which lack PNP phosphatase activity (123,161,165). These findings may reflect basic differences in the structural requirements for activity among these enzymes. It appears that alkaline and acid phosphatases will act upon compounds resembling PNPP, regardless of the context in which these structures are found (ie, as free phosphoesters or protein-bound), whereas

Table 23 Phosphotyrosyl-Protein Phosphatases

Tissue	Subcellular Location	M_r	Substrate	PNPP activity
A431 cells		?	EGF-rec.	?
Rat cells	P.M.[a]	?	vesicle proteins	
E coli	P.M.		histone, EGF-rec.	high
Calf Intestine	P.M.			high
Bovine Liver	P.M.			high
TCRC-2 cells	P.M.	?		high
Rat muscle	cytosol	?	IgG	
Rat liver				
Human Astrocytoma	P.M.	?	histone	high
EAT cells		?	glutamine synthase	
EAT cells	cytosol	40K	CM-SC[b] phosphorylase	low
Chicken Brain	cytosol	30-70K	casein	low
		43K	casein	low
		95K	casein	low
Bovine Heart	cytosol	13K	IgG	high
		65K	IgG	low
		95K	IgG	?

certain (P)Tyr-protein phosphatases may have stricter structural requirements for activity.

Extracts of mammalian tissues contain (P)Tyr-protein phosphatase activity which is inhibited by divalent cation and which is unaffected by inhibitors of alkaline phosphatase. These features clearly distinguish the primary (P)Tyr-protein phosphatases from the alkaline phosphatases. However, the alkaline phosphatases have been considered possible candidates as endogenous (P)Tyr-protein phosphatases for the following reasons: (a) Like many of the Tyr-protein kinases and their substrates, these enzymes are associated with the plasma membrane; (b) their optimal pH for dephosphorylating (P)Tyr-proteins is about 7; (c) in vitro, these enzymes dephosphorylate physiological (P)Tyr-proteins, such as the EGF receptor, but are relatively inactive toward (P)Ser/Thr-proteins (156). It should be remembered, however, that although most Tyr-protein kinase activity is membrane-associated, at least some of the prominent substrates for this activity are cytosolic (enolase, phosphoglycerate mutase, and lactate dehydrogenase) (84). Both the EGF and insulin receptors are internalized following binding of their respective ligands (67,195) and it has been suggested that other, as yet unidentified substrates, will also prove to be soluble

proteins.

Although the alkaline phosphatases do not appear to have a major role in (P)Tyr-protein phosphatase activity which can be extracted from the cytosol, a careful assessment needs to be made of the total (P)Tyr-phosphatase activity in various subcellular compartments. Preliminary data from this (Results 4.3.2) and other (D. Tabarini, personal communication) laboratories indicates that a significant amount of the total (P)Tyr-protein phosphatase activity in various tissues is associated with the particulate fraction. While it is not known whether this activity is associated with acid or alkaline phosphatases, such an association has previously been observed in membranes of human astrocytoma and TCRC-2 cells (157,158). In addition, the membrane fraction from hepatocytes has been shown to contain a Mg^{2+}-dependent, PNPP-inhibited, phosphatase which rapidly dephosphorylates the EGF receptor (196). Based on these data, it appears likely that this enzyme will also prove to be associated with an alkaline phosphatase.

The role of acid phosphatases as (P)Tyr-protein phosphatases in particular has to be carefully considered, as these enzymes appear to be highly specific for (P)Tyr-proteins, and may represent a major (P)Tyr-protein phosphatase in the cytosol. Indeed, with ^{32}P-Tyr-IgG as

substrate, about 80% of the total activity (measured at pH 5.0) in bovine heart extracts can be attributed to acid phosphatase. The isolated enzyme is almost completely inactive toward a variety of (P)Ser/Thr-proteins. Whether this specificity merely reflects a general activity toward all PNPP-like compounds or represents a physiologically important (P)Tyr-protein phosphatase activity cannot at present be determined.

5.2 Activity of Protein Phosphatases-1-4 toward Phosphotyrosyl-Proteins

Partially purified protein phosphatases-1-4 appear to have relatively little to no activity toward (P)Tyr-proteins (Refs. 159,197,198 and Results, 4.1.1 to 4.1.4). These enzymes will henceforth be designated (P)Ser/Thr-protein phosphatases to indicate their preferred substrates. Those species that do display activity toward (P)Tyr-proteins also possess readily measurable PNP phosphatase activity. The catalytic requirements for dephosphorylating (P)Tyr-proteins parallel those for PNP phosphatase activity even when these requirements differ from those for dephosphorylating (P)Ser-proteins. For example, the ^{32}P-Tyr-IgG phosphatase activity of (P)Ser-protein phosphatase-3A displays similar sensitivities to thermal degradation and to inhibitors as the PNP phosphatase activity of this enzyme, as well as

sharing the requirement of Mg^{2+} and reducing agents for maximal activity, while the properties of the phosphorylase phosphatase activity differ markedly (Results 4.2.3).

Although these enzymes are all much more active towards (P)Ser/Thr-proteins than toward the (P)Tyr-proteins tested, it remains possible that other (P)Tyr-proteins will reveal higher activity levels, or that even this low level of activity may have important physiologic consequence. Also, in addition to directly dephosphorylating (P)Tyr-proteins, the (P)Ser-protein phosphatases may influence tyrosine phosphorylation levels by modulating Tyr-protein kinase activity. Most of the Tyr-protein kinases are phosphorylated *in vivo* at both serine/threonine and tyrosine. The latter modification is thought to represent an autophosphorylation while the former may be catalyzed by cAMP-dependent protein kinase ($pp60^{src}$) (25,199), Ca^{2+}/phospholipid-dependent "C" kinase (EGF receptor/kinase) (200) and perhaps other protein kinases. In the case of the EGF receptor/kinase, threonine phosphorylation catalyzed by the C kinase decreases Tyr-protein kinase activity *in vivo* (200), while the Tyr-protein kinase activity of $pp60^{src}$ is augmented following phosphorylation of this enzyme at serine by cAMP-dependent protein kinase (201). It is possible that the (P)Ser/Thr-protein phosphatases play a role in regulating Tyr-protein kinase activity by acting upon these

sites of phosphorylation.

5.3 Phosphotyrosyl-Protein Phosphatases from Tissue Extracts

There is as yet no consensus as to the relationship between the various (P)Tyr-protein phosphatases in tissue extracts. Since these enzymes were isolated from different sources and were identified using different substrates, it is not surprising that different results have been obtained. There are, however, certain features common to these tissues. Extracts of Ehrlich Ascites Tumor cells, chicken brain, and bovine heart (assayed using ^{32}P-Tyr-phosphorylase, -casein, and -IgG, respectively) each contain multiple (P)Tyr-protein phosphatase isozymes separable by anion-exchange chromatography. A significant amount of the total activity in these extracts does not bind to DEAE at neutral pH, in contrast to (P)Ser-protein phosphatase activity, which is almost wholly retained by the resin. About half of the (P)Tyr-phosphatase activity elutes between 0.1-0.2 M NaCl. In chicken brain and bovine heart a third isozyme, eluting at about 0.3-0.4 M NaCl, has also been described. In the following sections, these enzymes will be referred to as Y-1, -2, and -3, for the sake of clarity, but this nomenclature is only used to indicate the relative strength of interaction of these (P)Tyr-protein

phosphatases with DEAE, and does not imply that they are similar in any other respect.

5.3.1 Phosphotyrosyl-Protein Phosphatase Y-1

The (P)Tyr-protein phosphatase activity which does not bind to DEAE (Y-1) is likely to be composed of several enzymes. In chicken brain this activity displayed a broad elution profile on gel filtration, ranging from M_r 30,000 to 100,000 (161). In Ehrlich Ascites Tumor cell extracts, the majority of this activity eluted from Sephacryl S-300 at a position corresponding to M_r 40,000 (165). It should be noted, however, that a smaller molecular species of M_r 15,000-20,000 was consistently observed during gel filtration which could represent either a proteolytic fragment of the M_r 40,000 species or an unrelated phosphatase. The DEAE-breakthrough fraction of (P)Tyr-protein phosphatase activity from bovine heart (^{32}P-Tyr-IgG as substrate) has an acidic pH optimum and co-purifies with acid phosphatase activity. This enzyme has an M_r of 13,000 and is probably identical to a low molecular weight acid phosphatase previously described in bovine liver and brain (Refs. 100-102 and Introduction 1.2.1.1) Thus, many differences and few similarities have been uncovered concerning the Y-1 phosphatases. Although these enzymes were isolated from different sources, there is evidence that

at least some of the observed differences may be attributed to the substrates used in these studies. For example, when the bovine heart extract was re-examined using ^{32}P-Tyr-casein as substrate in place of ^{32}P-Tyr-IgG, the (P)Tyr-protein phosphatase activity in the DEAE-breakthrough fraction displayed a neutral pH optimum and preliminary data indicates that this activity behaves as a M_r = 40,000 species on gel filtration [1]. These data suggest that bovine heart contains at least two (P)Tyr-protein phosphatases which do not bind to DEAE; a small enzyme which preferentially dephosphorylates ^{32}P-Tyr-IgG and which co-purifies with acid phosphatase activity, and a larger species with preferential activity toward ^{32}P-Tyr-casein. The substrates themselves are unlikely to dictate the pH optimum for activity, as the partially purified M_r 13,000 phosphatase optimally dephosphorylates both ^{32}P-Tyr-casein and ^{32}P-Tyr-IgG at pH 5.0, while the partially purified bovine heart Y-2 enzyme (M_r 65,000) optimally dephosphorylates these substrates at pH 6.5-7.0.

5.3.2 Phosphotyrosyl-Protein Phosphatase Y-2

The (P)Tyr-protein phosphatase activity eluting from DEAE at 0.1-0.2 M NaCl (Y-2) has been partially purified from

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1. J. Chernoff and H. C. Li, unpublished observation

Ehrlich Ascites Tumor cells, chicken brain, and bovine heart, and molecular weights of 40,000, 43,000, and 65,000, respectively, have been reported (123,161,165). These enzymes have a neutral pH optimum for activity, preferentially dephosphorylate (P)Tyr-proteins, and have little PNP phosphatase activity. The chicken and bovine enzyme are inhibited by Mg^{2+} and Mn^{2+} and stimulated by EDTA, while the tumor cell enzyme is inhibited by Zn^{2+}. Although sharing certain properties, it is not known whether these enzymes are related species.

5.3.3 Phosphotyrosyl-Protein Phosphatase Y-3

The (P)Tyr-protein phosphatase activity which elutes from DEAE-cellulose at relatively high salt concentrations (Y-3) has been reported to have a molecular weight of 95,000 in extracts of chicken brain (161). In bovine heart extracts, this activity peak co-elutes from DEAE-cellulose with (P)Ser-protein phosphatase-3C (Fig. 15b, 15b). As partially purified (P)Ser-protein phosphatase-3C has an M_r of 95,000 and has been demonstrated to possess activity toward (P)Tyr-proteins, it is possible that the two enzymes are similar. However, in chicken brain the Y-3 enzyme dephosphorylated ^{32}P-Tyr-casein six times faster than ^{32}P-Ser-casein and was stimulated by EDTA; two features which clearly distinguish this enzyme from (P)Ser-protein

phosphatase-3C. The author's laboratory has not pursued the purification of phosphatase Y-3, but the available data suggest that this enzyme is distinct from (P)Ser-protein phosphatase-3C despite their co-elution on DEAE-cellulose.

5.4 Inhibitors of Phosphotyrosyl-Protein Phosphatases

5.4.1 Protein Inhibitors

As heat-stable protein inhibitors exist for (P)Ser-protein phosphatase-1, it is possible that similar proteins will be found which inhibit (P)Tyr-protein phosphatases. If such proteins exist, they are likely to be different than inhibitors-1 and -2 from rabbit skeletal muscle, as these inhibitors have been shown to have little effect on crude or partially purified (P)Tyr-protein phosphatases (123,164). Hemmings et al (202) have reported that a heat-stable, nondialyzable inhibitor of (P)Tyr-protein phosphatase activity is present in boiled extracts of Ehrlich Ascites Tumor cells, but little data are available concerning this putative inhibitor.

5.4.2 Zn^{2+}, Vanadate, and Molybdate

Both Zn^{2+} (157,162-165) and vanadate (123,158,159,203) have been described as specific inhibitors of (P)Tyr-protein phosphatases. The data regarding Zn^{2+}, however, should be treated with caution. While it is true that Zn^{2+} has been

shown to inhibit certain endogenous and isolated (P)Tyr-protein phosphatase activity, Zn^{2+} is also a potent inhibitor of many (P)Ser-protein phosphatases [2]. The EDTA-stimulated (P)Tyr-protein phosphatases reported by this (141) and other (161) laboratories may be a similar enzyme to the Zn^{2+}-inhibited enzyme described by Horlein et al (165), but no data are available at this time implicating Zn^{2+} as a specific inhibitor of these EDTA-stimulated enzymes.

Vanadate is known to be a potent inhibitor of several enzymes concerned with phosphorylation and dephosphorylation, such as plasma membrane ATPases (204), acid and alkaline phosphatases (205-207), phosphofructokinase (208), and adenylate kinase (209). As mentioned previously, vanadate has also been shown to be a potent and selective inhibitor of (P)Tyr-protein phosphatases. These findings may account for some of the observed effects of this compound on intact cells. For example, vanadate interacts synergistically with EGF to enhance DNA synthesis in human fibroblasts (210). It has also been reported to mimic the effects of insulin on isolated rat hepatocytes (211,212). In light of the stimulation of Tyr-protein kinase activity caused by EGF and

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2. J. Chernoff and H. C. Li, unpublished observations

insulin and the inhibition of (P)Tyr-protein phosphatase activity caused by vanadate, it is tempting to speculate that some of the common intracellular effects of these compounds are mediated by increased tyrosine phosphorylation

Molybdate appears to be an extremely potent inhibitor of the (P)Tyr-protein phosphatase activity associated with prostatic acid phosphatase and the M_r 13,000 bovine heart Y-1 enzyme. As both these enzymes are acid phosphatases, it is possible that the inhibition by molybdate will be unique to this group of enzymes. The effects of molybdate on other (P)Tyr-protein phosphatases have not been reported.

5.5 Conclusions

Unlike the protein kinases, which phosphorylate proteins either at serine or threonine residues or at tyrosine residues exclusively, most protein phosphatases appear not to be absolutely specific as to the phosphoamino acid present in their substrates. However, they do exhibit a marked preference for either (P)Ser- or (P)Thr-proteins on the one hand, or (P)Tyr-proteins on the other. Thus, in analogy to the protein kinases, the protein phosphatases may be thought of as divided into two classes. These classes seem to be composed of distinct, unrelated enzymes, but more detailed studies are needed to confirm this impression. Although the precise circuits through which Tyr-protein

kinases influence cellular processes are not as well defined as those involving Ser/Thr-protein kinases, it is likely that the paths of serine/threonine and tyrosine phosphorylation are intricately intertwined. For example, as indicated earlier, evidence is accumulating that the activities of certain Tyr-protein kinases are directly affected by phosphorylation at serine or threonine (200,201). Also, nearly all the substrates for Tyr-protein kinases identified *in vivo* have been shown to contain phosphoserine and/or phosphothreonine in addition to phosphotyrosine (for a review, see Ref. 69) suggesting that these proteins may be modulated by both classes of protein kinase, and, by extension, both classes of protein phosphatase.

The physiological role of alkaline and acid phosphatases *vis-a-vis* the protein phosphatases in dephosphorylating (P)Tyr-proteins cannot at present be assessed. Such a determination might be made if (a) physiologically important substrates for Tyr-protein kinase activity were identified and (b) selective inhibitors or activators of the various enzymes active towards (P)Tyr-proteins were available. In that case, cellular extracts could be examined for relative levels of activity of individual (P)Tyr-protein phosphatases, and these activities could be monitored during events in which tyrosine dephosphorylation is thought to be

critical, e.g., upon reversion from the transformed to the normal phenotype in cells infected with ts RSV mutants.

Although the effects of tyrosine phosphorylation are not precisely defined, abundant evidence suggests that it is involved in the control of cellular growth. In order to understand this process, therefore, the enzymes which control the level of tyrosine phosphorylation should be thoroughly investigated. The study of the (P)Tyr-protein phosphatases, in particular, is but in its infancy, and much remains to be discovered concerning the structure, regulation, and function of these important enzymes.

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