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THE PROTEIN PHOSPHATASE PROPERTIES OF HUMAN LIVER ALKALINE PHOSPHATASE

by

(C) JOHN RAPHAEL ALLUM CHAN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled

THE PROTEIN PHOSPHATASE PROPERTIES OF HUMAN LIVER ALKALINE PHOSPHATASE

submitted by JOHN RAPHAEL ALLUM CHAN

in partial fulfillment of the requirements for the degree

of MASTER OF SCIENCE

in EXPERIMENTAL PATHOLOGY

.....
Supervisor

.....

.....

Date: May 14 / 1986

In Memoriam

Frater Lawrencus (Presentation College, Trinidad), magister et amicus meus

'Laboria omnia vincit'

ABSTRACT

Purified alkaline phosphatase and plasma membranes from human liver were shown to dephosphorylate phosphohistones. Neither plasma membranes nor purified liver alkaline phosphatase dephosphorylate glycogen phosphorylase a. The histone phosphatase activity of the liver plasma membranes was inhibited by levamisole, a specific inhibitor of alkaline phosphatase, and by phenylphosphonate and orthovanadate, but was relatively insensitive to fluoride. Autophosphorylation of plasma membrane proteins (by endogenous protein kinase(s)) was enhanced by levamisole, phenylphosphonate and orthovanadate. Also, the dephosphorylation of plasma membrane phosphoproteins by the endogenous protein phosphatase(s) was inhibited by levamisole; these observations suggested the involvement of membrane alkaline phosphatase.

Autophosphorylation of the plasma membrane proteins was dependent on cyclic AMP. The membrane phosphoproteins were resolved by one- and two-dimensional polyacrylamide gel electrophoresis. The membrane phosphoproteins were visualized by autoradiography and three of these, with molecular weights of 74, 45 and 36 kDa, appeared to be the major substrates of the membrane protein kinase(s).

Phospholipase C_{II} had no activation effect on the endogenous protein kinase activity. However, plasma membranes, incubated with phospholipase C_{II} to

enzymatically remove the membrane alkaline phosphatase, showed increased protein phosphorylation and a corresponding decrease in the endogenous protein phosphatase activity.

It appears that alkaline phosphatase is the major protein phosphatase present in the liver plasma membranes. The presence of cyclic AMP-dependent protein kinases and alkaline phosphatase in the plasma membranes is consistent with the involvement of these enzymes in the mediation of the effect of cyclic AMP on the function of membrane proteins by reversible protein phosphorylation.

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LIST OF ABBREVIATIONS

ATP	Adenosine 5'-triphosphate
CHAPS	3-[(3-Chloroamidopropyl-dimethylammonio]-1-propanesulfonate
Cyclic AMP	Adenosine 3':5'-cyclic phosphate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid
FMA	Fluoresceine murcury acetate
Levamisole	L[-]2,3,5,6-Tetrahydro-6-phenylimadazo[2,1-b] thiazole
MES	2[N-Morpholino]ethane sulfonic acid
MOPS	3[N-Morpholino]propane sulfonic acid
NMR	Nuclear magnetic resonance
pNPP	p-Nitrophenylphosphate
PMSF	Phenylmethyl sulfonyl fluoride
SDS	Sodium dodecyl sulfate
SDS-PAGE	Polyacrylamide gel electrophoresis in the presence of SDS

CHAPTER ONE

THE ALKALINE PHOSPHATASE PROTEIN

Alkaline phosphatases (orthophosphoric-monoester phosphohydrolase, alkaline optimum, EC 3.1.3.1) are a group of zinc-containing metalloenzymes. Many attempts have been made to define the physiological roles of these enzymes since the pathological significance of their elevated serum levels was first recognized (1). It has been proposed that protein phosphatases may have an important role in the regulation of cellular metabolism, transformation and proliferation (2-7). Certain characteristics of the protein phosphatase activity of alkaline phosphatase have been determined (8-11), and it is hoped that this information may offer some clue into the elucidation of the physiological function of the human enzyme. This chapter deals with a review of the physical, biochemical and enzymatic properties of alkaline phosphatase.

A. CATALYTIC PROPERTIES

1. Reaction mechanism

Alkaline phosphatase catalyses the transfer of the phosphoryl group from phosphomonoesters to either water (phosphohydrolysis) or to other hydroxyl-containing acceptors (transphosphorylation). In both cases, the reaction mechanism involves the formation of a phosphoryl-

enzyme intermediate (12) at a specific serine residue (13-16). The pH optimum of the phosphohydrolytic activity of alkaline phosphatase is in the alkaline range but protein phosphatase activity appeared to be at or near neutral.

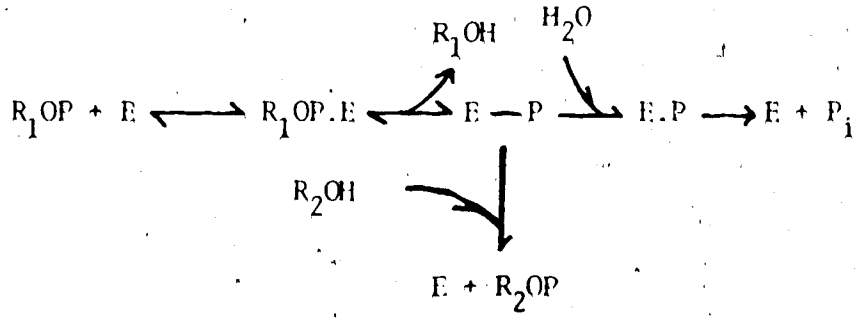

Fig. 1 Reaction scheme of alkaline phospahatase

In the reaction scheme shown in Figure 1, $R_1OP.E$ represents the Michaelis-Menten complex, E-P the covalent phosphoryl-enzyme, E.P the Michaelis-Menten complex for phosphate and enzyme, P_i inorganic phosphate, R_2OH a hydroxyl-containing molecule, and E an enzyme subunit (12).

2. Substrate specificity

Alkaline phosphatase is a non-specific phosphatase in that its rate of hydrolysis of the phosphate esters are similiar irrespective of the leaving group and there appears

to be no specific binding site for the alcoholic or phenolic portion of the substrate. The enzyme can hydrolyse the phosphate group from compounds with C-O-P, F-P, P-OP, S-P, or N-P type bonds (17). Derivatives of phosphonic acid with C-P bonds are not substrates of alkaline phosphatase.

Many of the phosphomonoesters which can be acted upon by alkaline phosphatase are physiologically important compounds. These include AMP, ATP, pyridoxal phosphate, carbamyl phosphate, phosphocholine, pyrophosphate, and phosphoethanolamine (17,18).

B. STRUCTURE AND MOLECULAR PROPERTIES

1. Molecular weight

The mammalian alkaline phosphatases are active as dimers (17). The molecular weights of the various isoenzymes varied from 119,000 Da for the placental enzyme to 180,000 Da for the kidney and liver forms (19).

There have been reports of tetrameric forms of alkaline phosphatase and this tetrameric structure has been ascribed to human placental, liver, and serum forms of the enzyme (18,20,21). However, it has been proposed that the 'tetramer' may represent a stable association of dimers of the enzyme or of an enzyme dimer tightly bound to other plasma membrane proteins (22). Human liver alkaline phosphatase was shown to be tetrameric in the plasma

membrane, consisting of four identical subunits (21). The evidence supports the tetrameric conformation of mammalian alkaline phosphatase in situ.

2. Amino acid composition

The amino acid composition of purified alkaline phosphatases from various bacterial and mammalian sources have been determined and minor differences in the amino acid composition among the various isoenzymes were found (23). The enzyme from Bacillus subtilis and B. licheniformis have unusually high lysine content (24,25) whereas the E. coli enzyme has very few aromatic amino acids but a large number of alanine residues (26).

Mammalian alkaline phosphatases tend to have more cysteine residues than the bacterial enzymes (27). Phenyl glyoxal, L-cysteine and FMA inactivated alkaline phosphatase (18,28) and it is likely that arginine and cysteine residues are involved in the reaction mechanism at the active site.

3. Protein structure

The alkaline phosphatase enzyme from E. coli is the best studied of this particular group of enzymes and much of the data collected from studies of the enzyme may be applicable to the other isoenzymes. The crystal structure of the bacterial alkaline phosphatase revealed each monomer (Fig. 2) to be made up of a large core of β-pleated sheets, seven

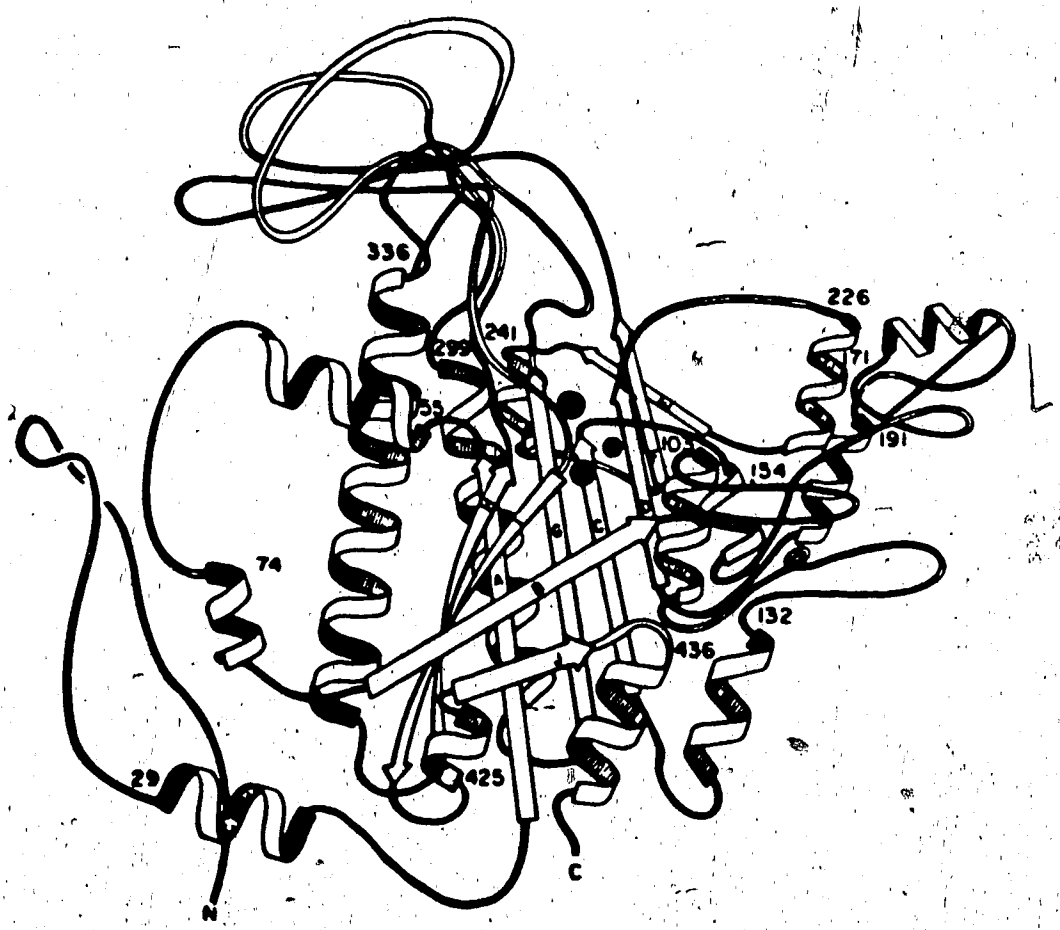

Fig. 2 Schematic of the secondary structure of E. coli alkaline phosphatase. The 3 functional metal sites represented as black circles, upper M1, lower M2, and M3 further away with a smaller radius. The numbers indicate the N termini (taken from ref. 31).

strands sandwiched between stretches of α-helices (29,30,31). Half of the residues are in α-helical segments with 13% and 16%, respectively, in β-strand and β-turn orientations (30). The helical segments of this α/β core represents 27% of the 459 amino acid residues of the E. coli alkaline phosphatase, with the β-strands making up 7%. The chains in the smaller domain are more convoluted. In both, there is extensive monomer-monomer contact (29). The active site pocket is located near the carboxyl ends of four strands and the amino end of the antiparrallel strand (31).

Bovine liver and human placental alkaline phosphatase have also been partially sequenced (14,32). Although the bovine enzyme was purified to apparant homogeneity with respect to SDS-PAGE, two different amino-terminal amino acid sequences were obtained (14). This suggests that the dimeric enzyme may be made up different subunits and hybrid human alkaline phosphatases may also exist (see 'Tumor isoenzymes').

4. Active site studies

Alkaline phosphatase catalysis has an essential requirement for Zn^{2+} for enzymatic function and this Zn(II)-enzyme can be activated by Mg^{2+} (33). Total reactivation of the apoenzyme upon addition of Zn^{2+} was not instantaneous, suggesting the presence of multiple metal-binding sites on the protein molecule (33). Removal of these extra weakly-bound metal ions did not appear to affect the enzymatic activity to any significant extent (34). The zinc ion at

the active site (Figure 3) can be replaced by Mn(II), Co(II), or Ni(II) ion although these enzymes were not as active as the normal Zn(II)-alkaline phosphatase (34).

X-ray crystallography of the protein molecule from E. coli showed the enzyme to be dimeric, with two zinc ions bound per dimer (34). There is a conformational change by the enzyme upon loss of the metal ions. The Zn^{2+} are located inside the enzyme's core, 3.9 Å apart (31), and the substrate molecules were found to be able to approach to within 3 Å of the metal ion (34).

NMR studies on the bacterial alkaline phosphatase with Cd^{2+} replacing the zinc ion revealed the presence of three Cd^{2+} binding sites, one of which corresponded to the active site (M1 site) (13,31). With increasing pH or upon phosphorylation of the enzyme, the cadmium ion migrated from the active site to another secondary site. The Cd(II)-alkaline phosphatase was found to have only 1% activity as compared to the normal Zn(II)-enzyme (13). The migration of the cadmium ion suggested possible conformational changes due to the change in pH or in the phosphorylation state of the serine residue at the active site. It must be noted that the cadmium ion is significantly larger than the zinc ion and that these findings may not be an accurate representation of normal Zn(II)-alkaline phosphatase (13).

The zinc ion at the active site (Fig.3) is complexed to one serine and three histidine residues (35). The NMR spectra indicated the presence of a fourth nitogen-

containing molecule at the active site but the identity of this amino acid is unknown. Water is involved at the active site, donating a proton to the phosphate group followed by hydrolysis of the phosphoserine group of the enzyme-phosphate intermediate (35). Comparison of the x-ray crystallographic data with the NMR findings, the water molecule is probably located at the M1 site (31). In the transphosphorylation reaction mechanism, the water is replaced by an alcohol or phenol.

The active site of the enzyme (from E. coli) may have a number of positively charged amino acids to neutralize and bind the negatively charged phosphate group; hydrolysis procedes with the attack on the O-P bond between serine-102 and the phosphate group (12). The zinc ion will also aid in binding of the phosphate group. The electrostatic forces involved in phosphate binding would explain the lack of substrate specificity of the alkaline phosphatases since a specific binding site for the nonphosphate portion of phosphomonoesters does not appear to exist (31,36). Arsenate, a product analog and enzyme inhibitor, binds between serine 102 and the zinc ion binding sites (31) sugesting the possible binding of the phosphate group of the substrate to the active site via a water molecule bridge (31,35).

The amino acid sequence of the active site for a few mammalian alkaline phosphatases have been also reported (14,32). Serine has been identified as the amino acid

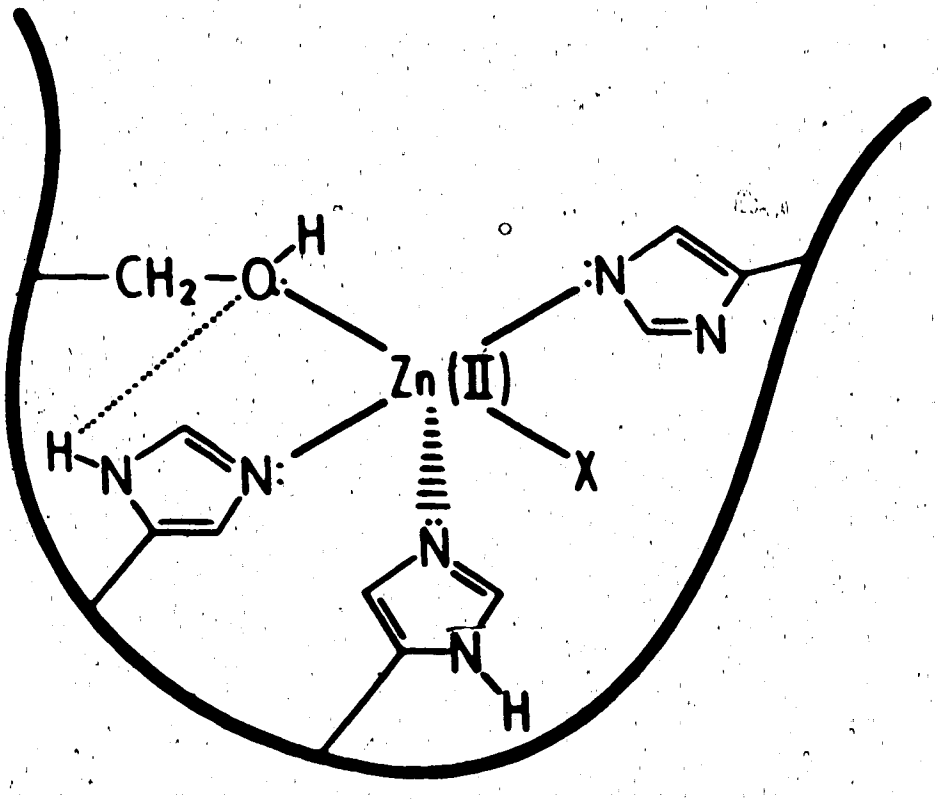

Fig. 3. Schematic of the active site of E. coli alkaline phosphatase (taken from ref. 35)

residue which forms the covalent phosphoryl-enzyme intermediate at the active site (14-16) and a sequence of Asp-Ser(P)-Ala for the bovine intestinal alkaline phosphatase was reported (14). Amino acid sequences for active-site tryptic peptides from human placental and bovine intestinal isoenzymes had 67% homology to each other, and 26% with the E. coli enzyme (14). The replacement of the active site serine by cysteine resulted in an active enzyme with some modification of its catalytic properties (37). It appears that catalytic function of alkaline phosphatase requires a neucleophile at the 102-position amino acid residue (37). Apparantly conservation of the active site sequence did not occur during the evolution of the enzymes nor does conservation appear to be needed.

5. Subunit interaction

^{31}P- and ^{113}Cd-NMR studies on bacterial alkaline phosphatase suggested possible negative cooperative interaction between the subunits upon phosphate binding (12). These findings have been supported by kinetic and ligand binding studies (38,39). The bacterial enzyme displayed non-Michaelian kinetics except under conditions of high ionic strength or high phosphate concentrations. The Lineweaver-Burk plots for the hydrolysis of p-nitrophenyl phosphate were non-linear and the analysis of the rate equation pointed to a negative cooperativity between the subunits of the alkaline phosphatase (38).

Binding studies with inorganic phosphate and L-phenylalanine showed the presence of two active sites per molecule of rat intestinal alkaline phosphatase (38,39). The binding affinity of the first occupied site was two orders of magnitude greater than that of the second occupied site, indicating a negative cooperativity between the subunits (39).

6. Carbohydrate content

Human alkaline phosphatases are glycoproteins and the properties of the isoenzymes appear to be partially determined by the carbohydrate groups on the protein molecules (40). Sialic acid is the major carbohydrate moiety present and it may be responsible for the heat stability and pronase resistance of the placental alkaline phosphatase isoenzyme.

The activation effect of magnesium ions on alkaline phosphatase appeared to be dependent on the mannose residues on the enzyme (41). Also, the metabolic half-life of the enzymes in the blood stream may be determined by the carbohydrate content (42).

C. HUMAN ALKALINE PHOSPHATASE ISOENZYMES

1. Introduction

Human alkaline phosphatase is found in the liver, bone, kidney, spleen, intestine, placenta and neutophils with

traces of the enzyme in other tissue (42-44). The enzyme is localized in the plasma membrane of the cells (18) although neutrophil alkaline phosphatase may be in a unique granule called a 'phosphasome' (45).

The alkaline phosphatases appear to be attached to the plasma membrane by a N-terminal transmembrane portion of the protein consisting of approximately 20 to 25 amino acid residues (46). Phosphatidylinositol may also be involved in the binding of the enzyme but the role of this phospholipid remains to be determined (47).

2. Genetic studies

Human alkaline phosphatases are coded for by at least three different genes (43). One gene codes for the liver/kidney/bone isoenzyme and possibly the neutrophil type (44), one for the intestinal, and a third for the placental isoenzyme (43). It has been proposed that a fourth gene is responsible for the fetal intestinal alkaline phosphatase (48).

The placental isoenzyme displays a high degree of polymorphism and it has been proposed that at least 18 alleles which coded for 44 phenotypes are present at the gene locus (49). Studies with monoclonal antibodies indicated a common epitope between the placental and the intestinal alkaline phosphatases with no similiarity to the non tissue-specific isoenzyme (liver/bone/kidney) (50).

Antibodies have also been used in the purification procedure of alkaline phosphatase as an affinity chromatographic step with the antibodies bound to a Sepharose gel base (51). The isolation of pure enzyme is a necessity for biochemical studies and use of monoclonal antibodies for enzyme purification appears promising.

3. Serum levels of alkaline phosphatase

Human serum alkaline phosphatase is a mixture of the various tissue-specific isoenzymes, mainly the liver type with some intestinal. Placental alkaline phosphatase is also found in serum during pregnancy (49). Abnormal levels of the enzyme in the serum reflect possible cellular or tissue damage, abnormal cellular function or cellular proliferation (52). The alkaline phosphatase may also enter the blood stream as a result of partial proteolysis or solubilization of the membrane enzyme by bile salts (42).

The various isoenzymes of alkaline phosphatase can be differentiated by heat inactivation, urea denaturation, electrophoresis or by the use of inhibition profiles (42). Human alkaline phosphatases were found to be inhibited to varying extents by simple amino acids (42). L-phenylalanine and L-homoarginine preferentially inhibited the intestinal and the liver isoenzymes respectively. This unique specificity of the amino acids with respect to inhibition of

the alkaline phosphatase isoenzymes meant that inhibition profiles could be set up and used to differentiate the isoenzymes (42).

Serum alkaline phosphatase determination has been of diagnostic value in renal diseases (53), diabetes mellitus (54), osteomalacia (55), ankylosing spondylytis (56), hepatobiliary and bone disorders (42,57-59) and tumors (see below).

4. Tumor isoenzymes

Alkaline phosphatase has been used as a tumor marker (60-62) and in many instances, a placental-like isoenzyme was found in the serum (49). It appears that an embryonic gene coding for the enzyme can be derepressed in certain tumors leading to the production of a variant of the placental alkaline phosphatase (42,49)

The first variant identified called the Regan isoenzyme, was found in the serum of a Peter Regan who had been diagnosed with bronchogenic cancer (42). This enzyme showed cross-reactivity with antibodies which were raised to placental alkaline phosphatase. The Regan isoenzyme, like the normal placental type, was heat stable, L-phenylalanine sensitive and its electrophoretic mobility was similiar to the normal placental form (42).

The Nagao isoenzyme was found in a patient with pleural carcinomatosis (63). Many characteristics of this variant were similiar to those of the normal placental alkaline

phosphatase but it also had unique properties: sensitive to inhibition by L-leucine, L-isoleucine and by EDTA (42).

A Regan-like enzyme was discovered in 1969 (64). Although this enzyme showed similiar biochemical properties as the Regan variant, it also showed sensitivity to inhibition by L-leucine and had greater heat stability than the normal placental enzyme (65). It appeared that certain enzymes may be hybrid alkaline phosphatases, made up of different subunits derived from the normal liver and placental alkaline phosphatases (66).

The Kasahara isoenzyme was first found in the serum of hepatoma patients (49). It resembles the meconium isoenzyme and its migration rate on electrophoresis was faster than the liver form.

D. PHYSIOLOGICAL ROLE OF ALKALINE PHOAPHATASES

1. Introduction

From certain biochemical and clinical studies, there is empirical evidence to indicate the importance of these enzyme at the cellular level. Some of the data will be reviewed here and theories related to the possible role of alkaline phosphatases will be discussed.

2. Phosphate transport

The alkaline phosphatase of many microorganisms is localized in the periplasmic space (67,68) and the enzyme is

associated with the uptake of inorganic phosphate. It has been shown that the synthesis of alkaline phosphatase by the organism was inducible by phosphate-poor media.

In vivo and in vitro studies with rat intestinal alkaline phosphatase indicated a similiar response by the intestinal tissues with respect to phosphate depletion, namely the enzyme activity increased (69,70). These findings lend support to the involvement of mammalian intestinal alkaline phosphatase in phosphate transport.

3. Hypophosphatasia

Hypophosphatasia is a rare hereditary bone disorder which is characterized by dental abnormalities, defective bone mineralization, and increased serum and urinary levels of phosphoethanolamine and inorganic pyrophosphate (42). It was also noted that the serum level of alkaline phosphatase was abnormally low with a significant decrease in the bone isoenzyme. The correlation between the alkaline phosphatase levels and the poor calcification process in the patients indicated a possible involvement of the bone isoenzyme in tissue mineralization (42).

However, a novel form of the disease was discovered in 1959 in which the patient displayed the classical signs of hypophosphatasia but the serum levels of alkaline phosphatase, though low, were within normal values (71,72). The serum alkaline phosphatase(s) behaved as expected with respect to routine biochemical testing with various

substrates (72). These findings raised doubts as to importance of the enzyme in bone formation. However, kindred studies have indicated that 'pseudohypophosphatasia' may be another genetic but milder form of familial hypophosphatasia (71).

Alkaline phosphatase may be involved in the mineralization of bone tissue by its catalytic breakdown of the inorganic pyrophosphate into a form which can be utilised by the osteoblasts (42).

4. Regulation of protein activity

The complexity and diversity of the reactions occurring in a living organism are aptly illustrated by the metabolic charts on the walls of biochemical laboratories. It would be logical to assume that the myriad of enzyme-catalyzed reactions are regulated in order for the organism to function normally. With the discovery of cyclic AMP-protein kinases (73) and subsequent findings with protein phosphatases (2), reversible phosphorylation was shown to be a major enzyme regulatory mechanism. Although the alkaline phosphatases have different physical and biochemical properties from the protein phosphatases (2), it has been known that the alkaline phosphatases can function as protein phosphatases.

Bacterial and mammalian alkaline phosphatases exhibit protein phosphatase activity acting on a wide variety of protein substrates. The enzyme from *E. coli* can

dephosphorylate glycogen synthetase, phosphohistones, phosphorylase kinase but not glycogen phosphorylase a (9). Similarly, human placental alkaline phosphatase dephosphorylated protamine, casein, phosvitin, phosphohistones and glycogen synthetase and the enzyme had a different pH optimum for each subatrate (10). A bovine intestinal isoenzyme was able to act on phosvitin and dentine phosphoproteins but the liver form cannot (73).

Whether alkaline phosphatase is intimately involved with respect to regulation of metabolic enzymes will require futher studies. A low molecular weight protein phosphatase (M_r = 35 kDa) from cardiac muscle and the adrenal cortex has been purified and characterized (75-77). This enzyme exhibited a significant p-nitrophenyl phosphate hydrolytic activity with a pH optimum of 8.5 and may be the catalytic subunit of the larger type 1 protein phosphatase (76). These findings indicate a possible relationship between protein phosphatases and phosphomonoesterases as represented by type 1 protein phosphatase and alkaline phosphatase respectively.

Alkaline phosphatase may also be involved in metabolic processes. Cellular maturation of X. laevis oocytes was inhibitable by low molecular weight phosphoesters and alkaline phosphatase could reverse the inhibition (78). Studies on cellular transformation and proliferation have implicated tyrosine-specific protein

kinases as the causative agents (3). Alkaline phosphatase was shown to dephosphorylate proteins which had been phosphorylated at tyrosine residues (8).

Protein kinases have been shown to be important components of the plasma membranes from platelets, erythrocytes, heart, muscle and other tissues (79-84). Membrane-bound protein phosphatases have also been reported (79,85-87). Alkaline phosphatase may be a member of this class of protein phosphatases by virtue of its membrane localization and its enzymatic activity, functioning as a protein phosphatase *in vivo* with the plasma membrane phosphoproteins as its substrates. The insulin receptor (88), glucocorticoid receptor (89) and spermidine N'-acetyltransferase (90) are examples of membrane proteins which are regulated by reversible phosphorylation.

E. PURPOSE OF THE STUDY

Much work has been done on the phosphomonoesterase activity of alkaline phosphatase (17,18,28). With the cellular role of general protein phosphatases established (2), a new approach to the task of determining the physiological significance of human alkaline phosphatase appeared feasible. Reports of the protein phosphatase activity of bacterial and certain mammalian alkaline phosphatases (4-10) prompted the need to characterize the possible protein phosphatase activity of the human enzyme.

The human alkaline phosphatases must function as a membrane-bound protein. Thus, we studied the protein phosphatase properties of liver plasma membranes of which alkaline phosphatase was an integral membrane protein. Also, if the physiological substrates of the enzyme are present in the plasma membrane, the membrane proteins must be separated and identified.

The aims of this study are to:

1. Identify and characterize the protein phosphatase activity of human liver plasma membranes using phosphohistones as the substrate ;
2. Assess the role of alkaline phosphatase in the dephosphorylation of plasma membrane phosphoproteins; and
3. Identify the physiological phosphoprotein substrate of the membrane alkaline phosphatase.

CHAPTER TWO

EXPERIMENTAL PROCEDURES

MATERIALS

Chemicals and biochemicals were from either Sigma Chemical Co. or Fisher Scientific Co. unless otherwise stated. All reagents used were of the highest purity available.

Chemicals used in the preparation of the polyacrylamide gels were from Bio-Rad Laboratories. Ampholytes, agarose, Sephadex G-25 and G-100 were from Pharmacia Fine Chemicals. DEAE cellulose was from Fisher Scientific.

[γ-^{32}P]ATP was from either New England Nuclear or Amersham Canada Limited. Nitrocellulose sheets and Whatman 3M filter paper were obtained from Fisher Scientific and x-ray film and film holders were from Picker International, Calgary, Canada.

Unless otherwise stated, the pH of all buffers was adjusted with HCl or NaOH solutions. Radiolabelled ATP was added to the buffers from the stock solution supplied by New England to the appropriate activity. All experiments and each assay of these experiments were performed at in duplicate.

METHODS

Liver Plasma Membrane Preparation

This was performed as described (18) with some modifications. Homogenization of the liver tissue was in 50 mM Tris-HCl, pH 7.5, with 0.1 % (w/v) PMSF. The purified plasma membranes were stored at -20°C in 100 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 4 mM $MgCl_2$, and 0.1 % (w/v) PMSF, until used.

Alkaline Phosphatase Assay

The assay was performed as described (19) with the assay buffer containing 10 mM pNPP and 1.5 mM $MgCl_2$ in 1 M 2-ethylamino ethanol, pH 10.3 . The rate of the reaction was monitored with a Varian 2200 recording spectrophotometer at 404 nm and calculation of activity was based on a molar absorption coefficient of 18700 for p-nitrophenol (44). One unit of enzyme activity corresponds to one mol of substrate hydrolyzed per min.

Protein Determination

Protein concentration was determined by the method of Markwell et al (91) with human serum albumin as the standard. This procedure was used since 1 % (w/v) SDS was present in the solution to aid in the solubilization of the plasma membrane proteins.

Preparation of the Phosphoprotein Substrates

1. ^{32}P-labelled histones. Histones (Type II S, 10 mg/ml) were incubated in 100 mM Tris-HCl, pH 7.5, with 4 mM $MgCl_2$, 0.4 mM [^{32}P]ATP, 5 μM cyclic AMP, and cyclic AMP-dependent bovine protein kinase (0.5 mg/ml). After 60 min at 37°C, 100 % (w/v) trichloroacetic acid solution was added to the mixture to a final concentration of 10 % (w/v). The suspension was centrifuged at 5000 x g for 10 min and the supernatant discarded. The precipitated histones were redissolved in the desired buffer and centrifuged at 5000 x g for 15 min to pellet the undissolved protein. The supernatant solution was saved and an equal volume of diethyl ether added and mixed to remove any remaining trichloroacetic acid. The aqueous layer containing the histones was removed and pH adjusted to the required value.

2. ^{32}P-labelled Phosphorylase a. Rabbit muscle phosphorylase b was dissolved (5 mg/ml, final concentration) in 50 mM Tris-HCL (pH 8.2), 50 mM sodium glycerol-2-phosphate, 1 mM [^{32}P]ATP, and 10 mM $MgCl_2$ (92). Phosphorylase kinase (0.03 mg/ml) was added to initiate the reaction which was carried out at 25°C for 60 min. The reaction was terminated by the addition of an equal volume of saturated ammonium sulfate and the protein suspension centrifuged at 5000 x g for 15 min. The precipitate was redissolved in the desired buffer and chromatographed on a

column of Sephadex G-50 to remove the salt. The column was equilibrated with, and protein eluted by, 100 mM Tris-HCl, pH 7.5 with 4 mM $MgCl_2$ present.

Purification of Phospholipase C_{II}

The starting material for the preparation of phospholipase C_{II} was obtained from Sigma (*B. cereus* phospholipase C, P-6135). The lyophilized powder (5 mg) was dissolved in 1 ml of 50 mM Tris-HCl (pH 7.6) with 10% (v/v) glycerol and applied to a column of Sephadex G-100 (1 x 30 cm), equilibrated and eluted with the above buffer as described (93). As both the non-specific phospholipase C and the phospholipase C_{II} eluted off the column together, the fractions (0.7 ml) were assayed for non-specific phospholipase activity as described (94). The fractions with this activity were pooled and subsequently loaded onto a column of DEAE-cellulose (1 ml bed volume) which had been equilibrated in the above buffer. The column was eluted with a gradient of NaCl (0.1 to 0.4 M) in the stated buffer with a 1.0 ml fraction volume. The phospholipase C_{II} activity was determined by its ability to release alkaline phosphatase from liver plasma membranes; equal volumes of the eluate and plasma membranes (20 mg/ml) were incubated for 15 min at 37°C. The suspension was centrifuged for 5 min at 100,000 x *g* in a Beckman Airfuge and the supernatant assayed for alkaline phosphatase activity as described above. Fractions which had phospholipase C_{II} activity were pooled and

dialysed for 24 h against a buffer of 50 mM Tris-HCl and 10% (v/v) glycerol. The enzyme was stored at -20°C until required.

Electrophorectic Transfer of Proteins

Proteins were transferred from polyacrylamide gels onto nitrocellulose sheets (Western Blot) as described (95). The procedure was carried out in a Trans Blot cell (Bio Rad Laboratories). The buffer was 25 mM Tris-HCl, pH 8.3, with 0.192 M glycine and 20 % (v/v) methanol and the blotting performed at 4°C under a constant current of 0.11 A for 20 h.

Drying of Polyacrylamide Gels

The gels were soaked in a solution of 50 % (v/v) methanol, 10 % (v/v) glycerol, and 5 % (v/v) acetic acid for 15 min. The gel was placed onto Whatman 3M filter paper and dried under vacuum at 80°C.

Colour Silver Staining of Polyacrylamide Gels

The slab gel (1.5 mm thickness) was fixed in a solution of 50 % (v/v) ethanol and 5 % (v/v) acetic acid for 20 h, with one change of solution (96). After fixing, the gel was rinsed in deionized water three times, each of 1 h. The gel was equilibrated for 30 min in 100 ml of a 0.2 % (w/v) solution of silver nitrate then rinsed once with water for

20 s. The proteins are developed by a solution of 7.5% (v/v) formaldehyde and 3% (w/v) NaOH.

Two-dimensional Separation of Phosphoamino Acids

[^{32}P]Phosphate-labelled proteins were precipitated with 15% (w/v) trichloroacetic acid (final concentration) and centifuged at 5000 x g for 10 min. The pellet was resuspended in 6M HCl and heated at 100°C for 90 min (97). After hydrolysis, the HCl was removed by evaporation and the sample lyophilized. Standards of phosphoserine, phosphothreonine and phosphotyrosine (5 μg each) were added to the hydrolysate and the amino acids separated by a combination of thin-layer electrophoresis and chromatography as described (97). The electrophoresis was at 1000 V for 90 min in 7.8% (v/v) formic acid and 2.5% (v/v) acetic acid on a thin-layer cellulose chromatogram sheet (Kodak). After electrophoresis and air-drying of the cellulose sheet, the chromatography was performed in a mixture of isobutyric acid and 0.5 M NH_4OH (5:3 ratio). The amino acid standards were visualized with ninhydrin [0.1% (w/v)] and the [^{32}P]phosphate-labelled amino acid determined by autoradiography.

CHAPTER THREE

THE PROTEIN PHOSPHATASE ACTIVITY OF PLASMA MEMBRANES

It has been shown previously that the plasma membrane-bound form of human liver alkaline phosphatase has significantly different kinetic and molecular properties compared to the soluble, purified enzyme (18). The membrane-bound form is probably tetrameric (21) and this may account for the differences in its enzymatic properties. It is likely that alkaline phosphatase carries out its physiological and biochemical roles as an integral membrane protein.

Protein phosphatases are involved in enzyme regulation by reversible phosphorylation reactions (2,73). Purified alkaline phosphatase is a protein phosphatase (11) and plasma membrane-bound alkaline phosphatase may also exhibit this activity. The plasma membranes of various eukaryotic cells have both endogenous protein kinase (80,81,110-112) and alkaline phosphatase activities (98,99). These two activities could work in concert to regulate the degree of membrane protein phosphorylation and control certain aspects of plasma membrane function.

An endogenous protein phosphatase activity was identified in human liver plasma membranes and its characterization will be described in this chapter.

EXPERIMENTAL PROCEDURES

Effect of Inhibitors on Protein Phosphatase Activities

The effects of orthovanadate (1 mM), levamisole (10 mM), phenyl phosphonate (10 mM), and KF (50 mM) on the histone phosphatase activity of purified alkaline phosphatase, plasma membranes and protein phosphatase 1 were determined. The assay was performed with inhibitor and enzyme in 4 mM $MgCl_2$, 100 mM Tris-HCl at pH 7.5; ^{32}P-labelled histones (5 mg/ml) were added to start the reaction. A reaction time of 5 min was used to determine the percent inhibition of the phosphatase activity. The reaction was terminated by the addition of trichloroacetic acid to a final concentration of 15 % (w/v) and the suspension was centrifuged at 5000 x g for 10 min. The supernatant was added to 3 ml of scintillation fluid (Scinti-Verse II, Fisher Scientific) to determine [^{32}P]phosphate released using an Isocap/300 Model 6868 Liquid Scintillation counter (Searle Analytical).

[^{32}P]Phosphate-labelled glycogen phosphorylase a was also used as a substrate though the effects of the inhibitors were not determined. The assay was performed as described for the phosphohistones above, but the reaction time was 10 min.

Effect of pH on Protein Phosphatase Activity

The release of [^{32}P]phosphate from phosphohistones by purified alkaline phosphatase or liver plasma membranes was

determined in 4 mM $MgCl_2$, 100 mM buffer salt and 5 mg/ml ^{32}P-labelled histones, with or without 10 mM levamisole. Plasma membranes (5 mg/ml protein, final concentration) or purified alkaline phosphatase (0.015 units of pNPP activity) were added to initiate the reaction of 10 min duration at 30°C.

Effect of Inhibitors on the Histone Kinase Activity of Cyclic AMP-dependent Bovine Protein Kinase

Histones (2 mg/ml) were phosphorylated by a cyclic AMP-dependent bovine protein kinase (13 μg/ml), with and without inhibitors in a reaction buffer containing 6 mM $MgCl_2$, 0.6 mM [^{32}P]ATP (0.1 Ci/mmol), 5 μM cyclic AMP and 150 mM Tris-HCl at pH 7.5 (112). Incubation was at 30°C for 30 min and the reaction was terminated by the addition of trichloroacetic acid to a final concentration of 15 % (w/v). The precipitate was filtered onto a Whatman GF/E filter, washed 5 times with 1 ml volumes of 10 % (w/v) trichloroacetic acid and air-dried. The filter was placed in a vial with 3 ml scintillation fluid for [^{32}P]phosphate determination.

Histones Kinase Activity of Human Liver Plasma Membranes

The activity of endogenous plasma membrane protein kinases was determined using histones as the protein substrate. The reaction was performed in 100 μl of reaction buffer, 1.0 mM orthovanadate, and 20. mg/ml histone with or

without 5 M cyclic AMP; plasma membranes (10 µl, containing 10 mg protein/ml) were added to start the reaction. Incubation was at 30°C for 0-90 min and reaction terminated by cooling the mixture on ice and immediately centrifuging it at 20,000 x g for 10 min to pellet the plasma membranes from the soluble histones. The supernatant was removed, the histones precipitated by trichloroacetic acid, the precipitate filtered and washed, and [^{32}P]phosphate incorporated determined as described above.

Plasma Membrane Protein Phosphorylation by Cyclic AMP-dependent Bovine Protein Kinase

Liver plasma membranes (5 mg/ml) were phosphorylated by a cyclic AMP-dependent bovine protein kinase (0.2 mg/ml) as described, with some modifications (80). The phosphorylation buffer was 0.2 mM [^{32}P]ATP (0.1 Ci/mmol), 4 mM $MgCl_2$, and 100 mM Tris-HCl, pH 7.5. Incubation was at 30°C for 0 - 60 min. Incorporation of [^{32}P]phosphate into the plasma membrane proteins was determined by trichloroacetic acid precipitation as were the effects of inhibitors on the bovine protein kinase activity as described above.

Autophosphorylation of Liver Plasma Membrane Proteins

Plasma membranes (10 mg of protein/ml) were autophosphorylated using the phosphorylation buffer, with or without cyclic AMP (5 µM). The effects of inhibitors on this

activity were also determined. Incubation was at 30°C for 0 - 120 min. [^{32}P]Phosphate incorporation was determined by trichloroacetic acid precipitation as described above.

Effect of Phospholipase C_{II} Treatment on Plasma Membrane Autophosphorylation

The plasma membranes were treated with the phospholipase C_{II} followed by washing of the membranes to remove any released alkaline phosphatase. Equal volumes of plasma membranes (20 mg/ml) and phospholipase C_{II} (2 µg/ml) were mixed and incubated at 37°C. At regular intervals, 100 l of the mixture was removed and centrifuged at 100,000 x g for 5 min. The supernatant was assayed for alkaline phosphatase activity as described above. When the alkaline phosphatase enzyme content remaining on the membranes has decreased to 50 % (as compared to activity at start of incubation), an aliquot was removed and the mixture cooled on ice followed by additions of 10 volumes of cold buffer (100 mM Tris-HCl, pH 7.5, and 4 mM $MgCl_2$). The remaining membranes were further treated until the alkaline phosphatase activity of these membranes has decreased to 5% and the reaction stopped as describes above. The plasma membranes were washed three times with the buffer and resuspended to a protein concentration of 10 mg/ml. These plasma membranes were autophosphorylated and [^{32}P]phosphate incorporation determined as described before.

Dephosphorylation of [^{32}P]Phosphate-labelled Liver Plasma Membrane Proteins

Plasma membranes (10 mg protein/ml) were autophosphorylated in the phosphorylation buffer described above except that 0.2 mM [^{32}P]ATP with a specific activity of 2.6 mCi/mmol was used. The mixture was incubated for 15 min at 30°C, then non-radiolabelled ATP plus $MgCl_2$ were added to a final concentration of 10 mM. Equal volumes of the mixture were removed at appropriate times and the plasma membrane proteins precipitated with trichloroacetic acid and the precipitate treated as described above for [^{32}P]phosphate determination.

In another procedure, plasma membranes were autophosphorylated using the phosphorylation buffer with 1 mM orthovanadate present. After incubation at 30°C for 30 min, the plasma membranes were washed three times in 4 mM $MgCl_2$, 1 mM orthovanadate and 100 mM Tris-HCl, pH 7.5, at 4°C and resuspended to a final protein concentration of 5 mg/ml. Dephosphorylation of the [^{32}P]phosphoproteins was initiated by the addition of 5 mM (final concentration) (R)-(-)epinephrine which complexes the orthovanadate. Equal volumes of the mixture were removed and treated as described above to determine the [^{32}P]phosphate remaining on the plasma membrane proteins.

RESULTS

The effects of fluoride, phenylphosphonate, levamisole and orthovanadate on the protein phosphatase activity of purified alkaline phosphatase, protein phosphatase 1 and liver plasma membranes are summarized in Table 1. To calculate the inhibition values, the experiments were done under conditions as close to initial rates as possible, using an incubation period of 5 min. The histone phosphatase activity of these plasma membranes is inhibited by fluoride, phenylphosphonate and orthovanadate, known phosphatase inhibitors (79,100-102). Levamisole, a specific inhibitor of alkaline phosphatase (103,104) and orthovanadate are potent inhibitors of the pNPP hydrolase activity of alkaline phosphatase, but less so of its protein phosphatase activity. The reverse is true of KF and phenylphosphonate. Levamisole had little effect on protein phosphatase 1. None of these compounds were good inhibitors of bovine protein kinase (TABLE 1).

Using ^{32}P-labelled histone as substrate, the inhibition profiles of the protein phosphatase activity of purified alkaline phosphatase and of the liver plasma membrane were similiar, although the pure enzyme was more susceptible to all inhibitors (Fig. 4, TABLE 1). Particularly in the case of the pure enzyme, the dephosphorylation reactions appeared to be biphasic, with a rapid initial rate of protein phosphatase activity. This may reflect the accessibility of

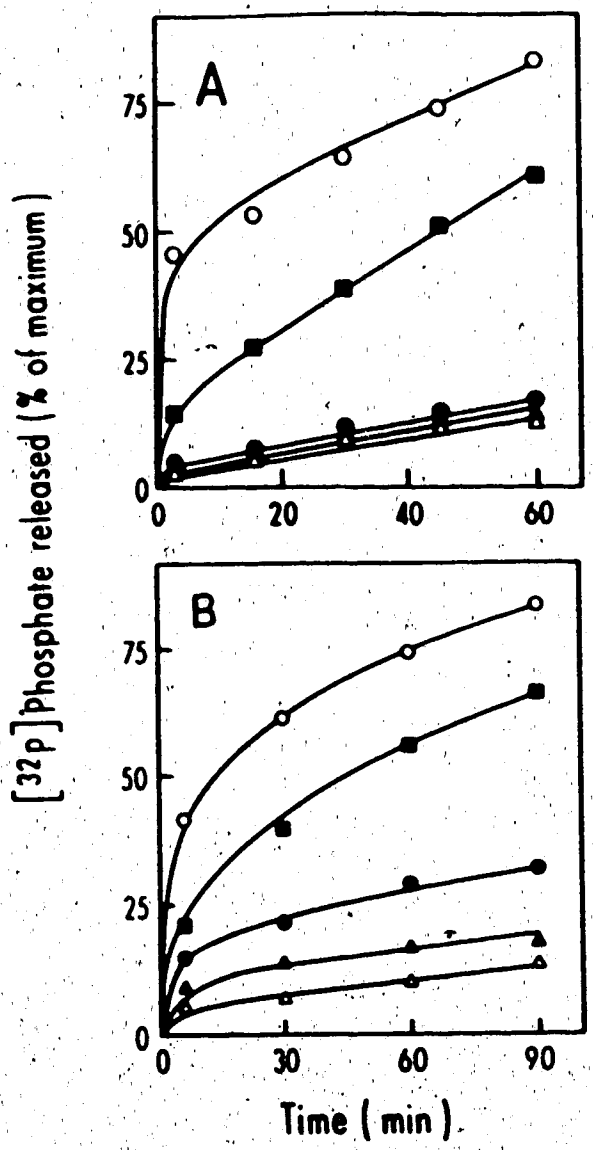

Fig. 4 Effect of inhibitors on histone phosphatase activity of purified alkaline phosphatase (A) and liver plasma membranes (B). The reaction buffer was 4 mM $MgCl_2$, and 5 mg/ml ^{32}P-labelled histones in 100 mM Tris-Hcl, pH 7.5. Additions were: none (○), 50 mM fluoride (■), 10 mM phenyl phosphonate (●), 10 mM levamisole (▲) or 1 mM orthovanadate (△).

TABLE 1. EFFECT OF INHIBITORS ON PROTEIN KINASE AND PHOSPHATASE ACTIVITIES

Protein kinase activity was determined in 0.6 mM [^{32}P]ATP, 6 mM $MgCl_2$, .2 mg/ml histones, 13 g/ml bovine protein kinase, 5 μM cyclic AMP, and 150 mM Tris-HCl, pH 7.5. Protein phosphatase activity was determined using 5 mg/ml ^{32}P-labelled histones and 4 mM $MgCl_2$ in 100 mM Tris-HCl, pH 7.5. Reactions were initiated by adddition of either enzyme or liver plasma membranes. The reaction time was 5 min.

Activities determined	Inhibition			
	KF (50 mM)	Phenyl phosphonate (10 mM)	Levam-isole (10 mM)	Ortho-vanadate (1 mM)
Hydrolysis of pNPP by alkaline phosphatase	13	47	94	97
Hydrolysis of pNPP by liver plasma membranes	10	45	96	98
Dephosphorylation of ^{32}P-labelled histiones by alkaline phosphatase	50	80	82	84
Dephosphorylation of ^{32}P-labelled histones by liver plasma membranes	45	61	76	79
Dephosphorylation of ^{32}P-labelled histones by protein phosphatase 1	70	75	15	75
Phosphorylation of histones by bovine protein kinase	38	37	10	14

the phosphoamino acid residues on the histones to the phosphatase and/or a degree of substrate heterogeneity. Nevertheless, it is clear that alkaline phosphatase in its native plasma membrane environment is still capable of dephosphorylating histone. Mg^{2+}, present for these experiments at 4 mM, is an activator of the protein phosphatase activity as well as the pNPP hydrolase activity (28).

The pH profiles of the histone phosphatase activity of purified alkaline phosphatase and of the liver plasma membranes were similiar (Fig. 5); both activities were optimal at pH 7.5 - 8.0. Addition of 10 mM levamisole to the reaction reduced the protein phosphatase activity of the plasma membranes to less than 22% at all pH values, and that of the purified alkaline phosphatase to less than 8%. This difference may be a reflection of the histone phosphatase activity in the membranes that is levamisole insensitive. The histone phosphatase activity of membrane alkaline phosphatase is less sensitive to pH than is the pure enzyme; at pH 5.0 the membranes still have 50% of the activity they had at the optimum, whereas the pure enzyme had only 15%. The same is true at pH 10.0, although the difference is less.

In the presence of orthovanadate to inhibit the endogenous protein phosphatases (TABLE 1), the liver plasma membranes exhibited a cyclic AMP-dependent protein kinase

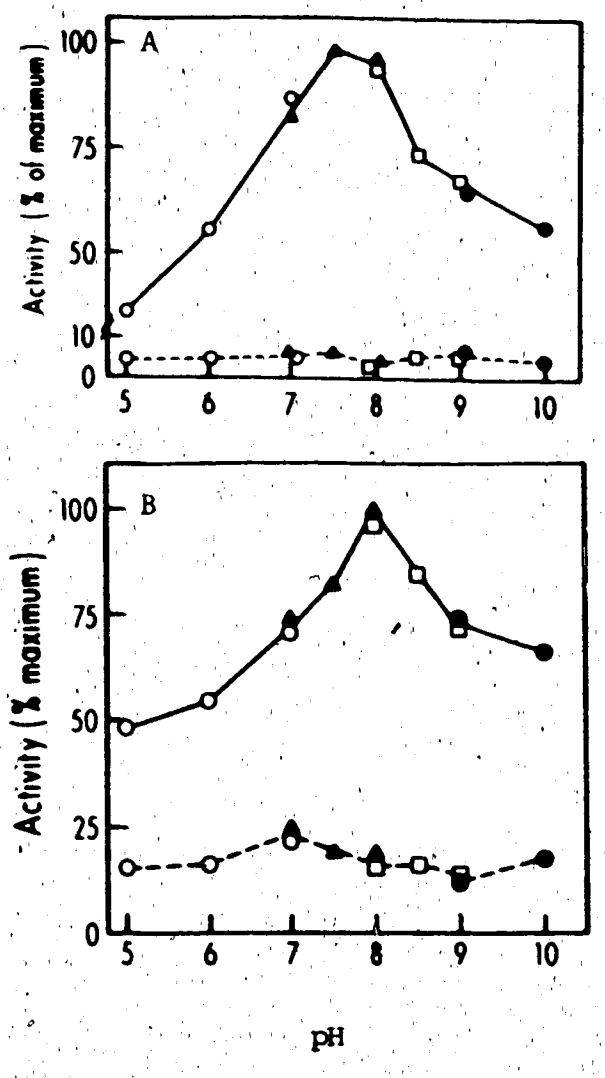

Fig. 5 Effect of pH on histone phosphatase activity of purified alkaline phosphatase (A) and liver plasma membranes (B). The medium was 4 mM $MgCl_2$, 5 mg/ml ^{32}P-labelled histone and 100 mM buffer salt. Buffers were: MES-HCl (O); MOPS-HCl (▲); Tris-HCl (■); and bicarbonate (●). No inhibitor (—) or 10 mM levamisole (---) added.

activity capable of phosphorylating an exogenous protein substrate (Fig. 6). Alkaline phosphatase, although capable of hydrolyzing free ATP, will not use Mg-ATP as a substrate, and indeed it is not even an inhibitor of the enzyme (28,43). Membrane proteins can also be phosphorylated by the cyclic AMP-dependent catalytic subunit of bovine protein kinase (Fig. 7). Both the rate and the degree of phosphorylation was enhanced by levamisole, orthovanadate and phenylphosphonate, an effect that could have resulted from either an activation of the kinase or an inhibition of the protein phosphatases. Since the phosphorylation of histones by bovine protein kinase is inhibited (and not activated) by these compounds (TABLE 1), it is likely that inhibition of the endogenous membrane protein phosphatases is responsible.

Endogenous protein kinases also catalyzed the incorporation of [^{32}P]phosphate from [^{32}P]ATP into the plasma membranes (Fig. 8); this autophosphorylation was also enhanced in the presence of levamisole and orthovanadate. Phospholipase C_{II} selectively catalyzes the release of certain enzymes including alkaline phosphatase from plasma membranes (93). Treatment of plasma membranes with phospholipase C_{II} to release the membrane alkaline phosphatase resulted in increased membrane protein phosphorylation (Fig. 9). The increased [^{32}P]phosphate incorporation seen is most likely due to decreased protein

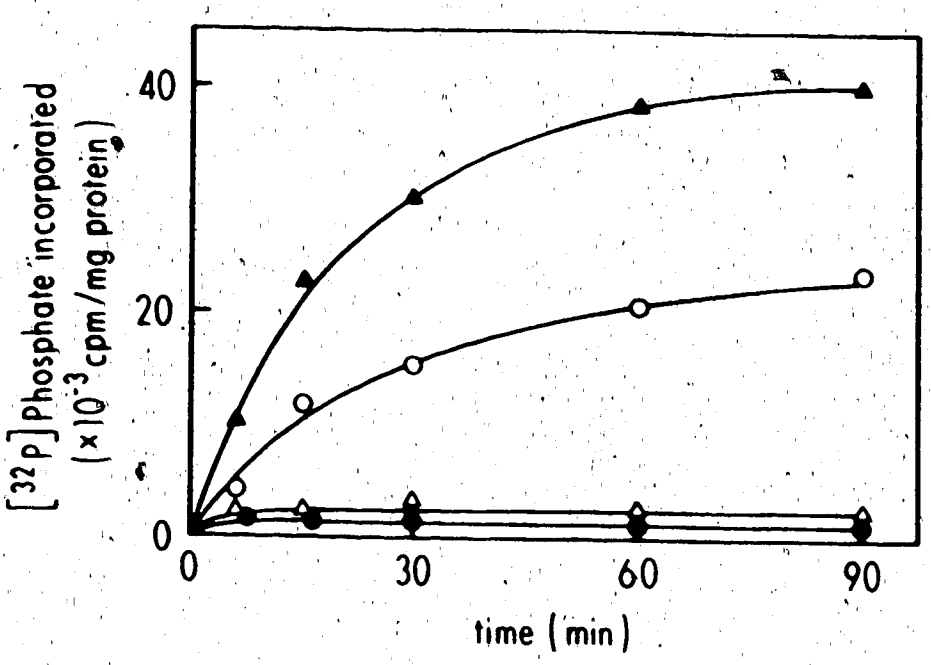

Fig. 6 Histone kinase activity of liver plasma membranes. The medium was 1 mM orthovanadate, 4 mM $MgCl_2$, 20 mg/ml histones and 0.2 mM [^{32}P]ATP in 100 mM Tris-HCl, pH 7.5. Additions were: none (●); plasma membranes only (△); plasma membranes and 5 μM cyclic AMP (○); bovine protein kinase and 5 μM cyclic AMP (▲).

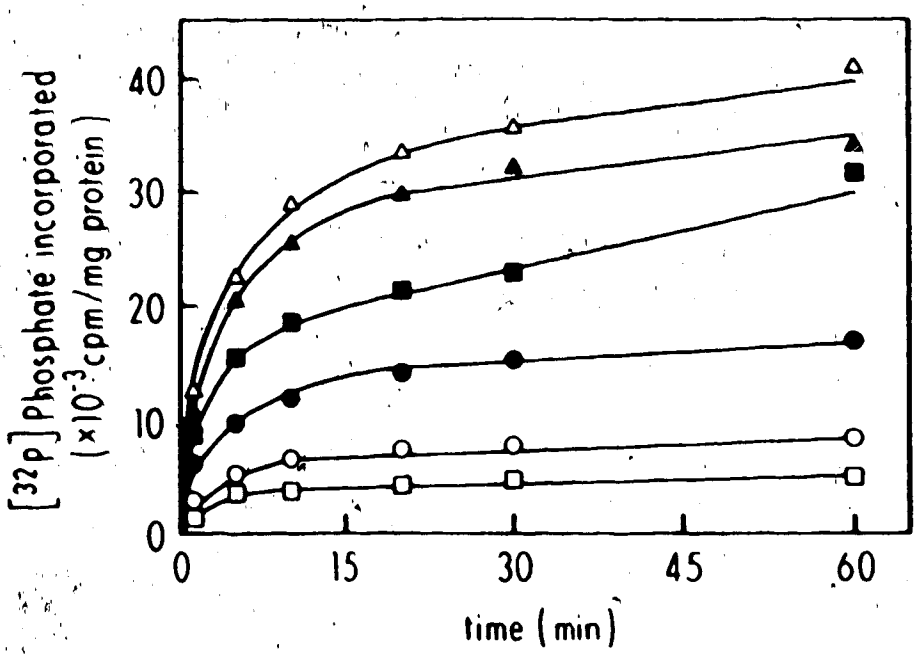

Fig. 7. The effect of inhibitors on the phosphorylation of plasma membrane proteins by cyclic AMP-dependent bovine protein kinase. The buffer was 4 mM $MgCl_2$, 8 mg/ml membrane protein, 5 μM cyclic AMP and 0.2 mM [^{32}P]ATP in 100 mM Tris-HCl, pH 7.5 (○). Protein kinase (0.2 mg/ml) was added to start reaction (●). The inhibitors were: 50 mM fluoride (□); 10 mM phenyl phosphonate (■); 10 mM levamisole (▲); 1 mM orthovanadate (△).

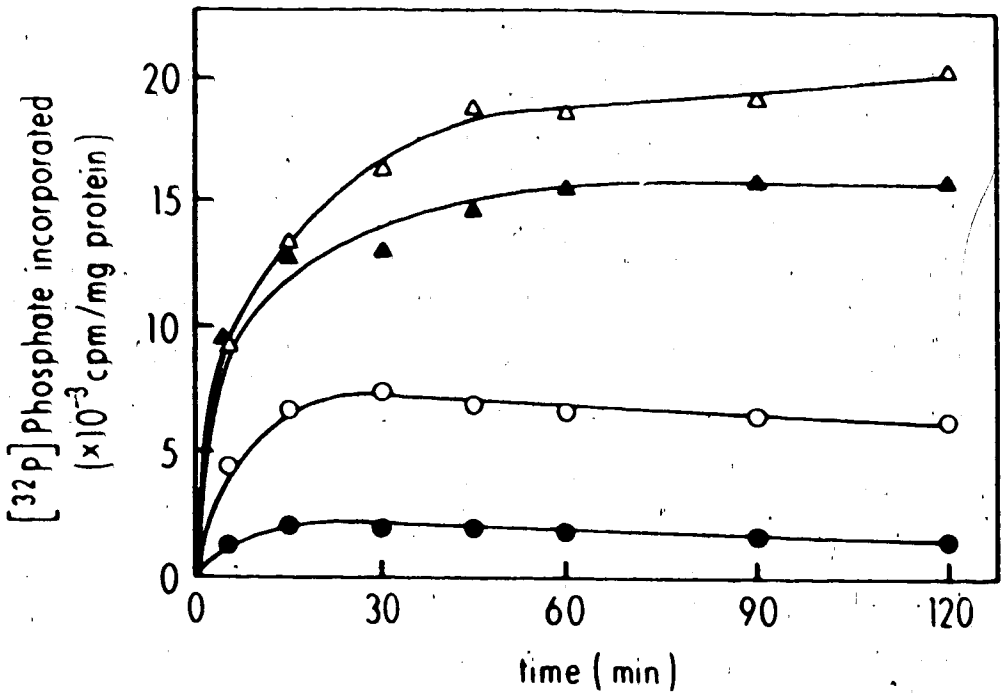

Fig. 8 Autophosphorylation of liver plasma membrane proteins

The phosphorylation was carried out in the buffer as described (O) or with the addition of 5 μM cyclic AMP (O); 5 μM cyclic AMP with 10 mM levamisole (▲); 5 μM cyclic AMP with 1 mM orthovanadate (Δ).

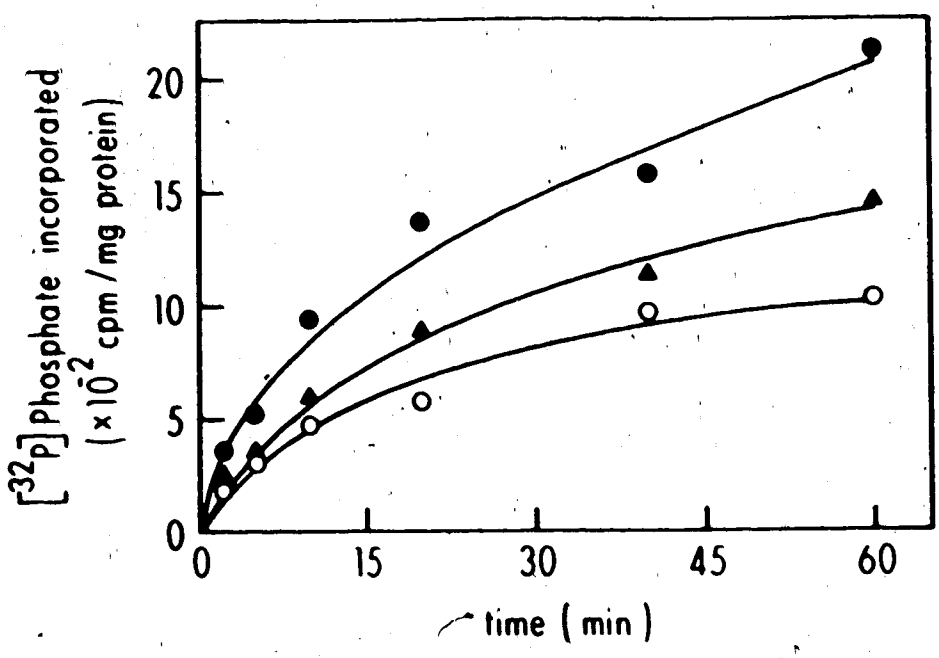

Fig. 9 Effect of Phospholipase C_{II} on the autophosphorylation of membrane proteins. The plasma membranes were treated with phospholipase C_{II} to remove the alkaline phosphatase by 50% (▲) or > 95% (●) and the membrane proteins autophosphorylated as described in text. Plasma membranes which were not treated with the phospholipase C_{II} were used as the control (○).

phospahatase activity from the loss of the membrane alkaline phosphatase (18).

To further investigate the nature of the inhibitor-enhanced phosphorylation of the plasma membranes, it was necessary to assess separately the protein kinase and phosphatase activities. This was achieved by the use of orthovanadate, an inhibitor of all phosphatases but not of bovine protein kinase (TABLE 1). The inhibitory effect of orthovanadate can be reversed by (R)-(-)epinephrine; This is due to chelation of the inhibitor by the amine (104). Plasma membrane proteins, phosphorylated by endogenous kinase in the presence of orthovanadate, sedimented and washed, were not dephosphorylated to any appreciable extent until (R)-(-)epinephrine was added (Fig. 10). Also this dephosphorylation could be inhibited by levamisole, the specific inhibitor of alkaline phosphatase, or accelerated by the addition of purified alkaline phosphatase. Kinase and phosphatase activities were also 'resolved' by carrying out the phosphorylation of the membrane proteins with [^{32}P]ATP then after 15 min, adding 10 mM non-radiolabelled Mg[ATP]. Phosphorylation with [^{32}P]ATP was essentially abolished and protein phosphatase activity can be followed by [^{32}P]phosphate release (Fig. 11).

Neither purified alkaline phosphatase nor plasma membranes had any activity towards glycogen phosphorylase a,

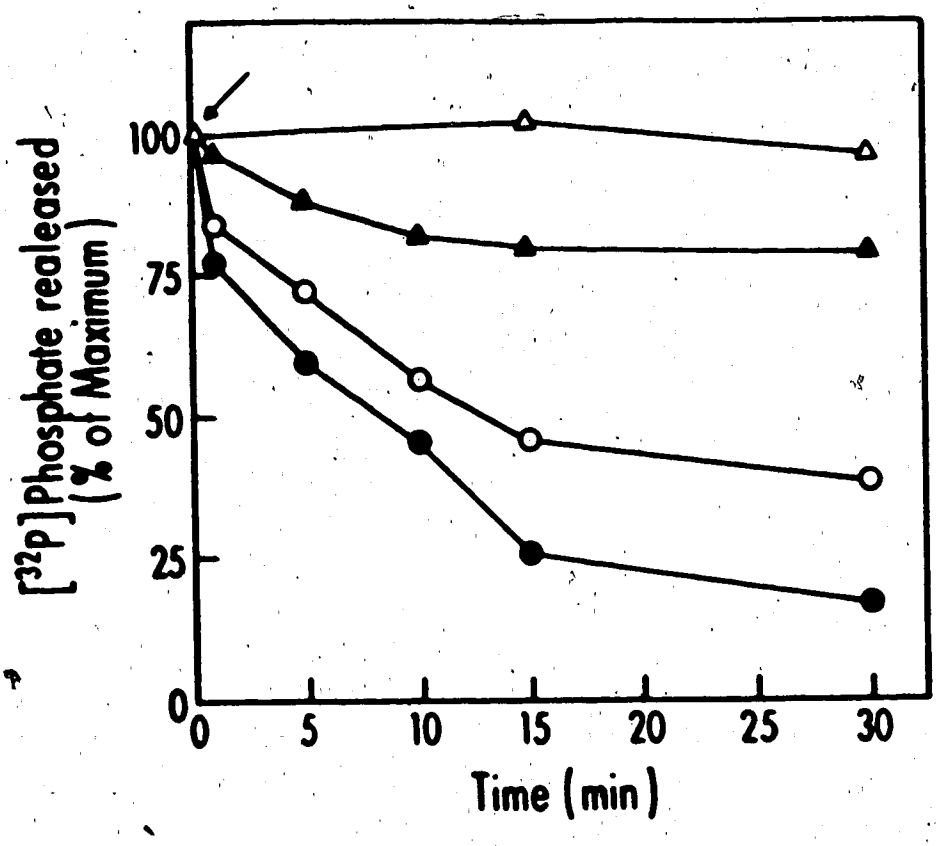

Fig. 10 Dephosphorylation of ^{32}P-labelled membrane phosphoproteins. Plasma membrane proteins were autophosphorylated as described in text. At zero time (arrow), (R)-(-)epinephrine was added. Curves are (R)-(-)epinephrine only (○); or with either 10 mM levamisole (▲); or 0.5 U liver alkaline phosphatase enzyme (●); Control (△).

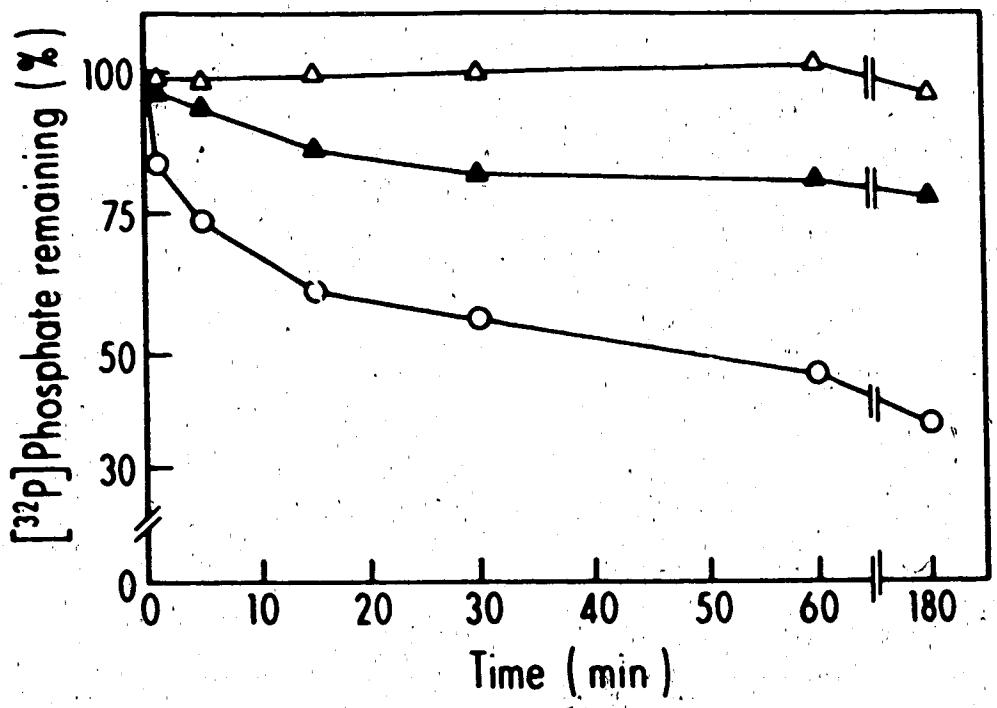

Fig. 11 Autodephosphorylation of membrane phosphoproteins. Plasma membrane proteins were autophosphorylated as described in text. At zero time, non radiolabelled Mg-ATP was added. The curves are: control (△); Mg-ATP plus 10 mM levamisole (▲); Mg-ATP only (○).

whereas protein phosphatase 1 dephosphorylated this substrate but not pNPP.

DISCUSSION

The findings suggest that alkaline phosphatase is a major protein phosphatase present in the liver plasma membranes, especially considering the fact that a specific inhibitor of the enzyme, levamisole, almost completely abolishes protein phosphatase activity. That the two activities are one and the same is supported by their similiar inhibition and pH profiles.

The release of alkaline phosphatase from rat liver plasma membranes by phospholipase C_{II} (18) suggested a possible relationship between alkaline phosphatase and the membrane phosphoinositol lipids. If the enzyme was attached to the plasma membrane by a covalent bond to the lipid molecule (47) it would explain the release of an intrinsic membrane protein by a phosphatidylinositol-specific phospholipase (phospholipase C_{II}) (18,21). Jemmerson et. al (46) suggested that human placental alkaline phosphatase is attached to the plasma membrane by a short transmembrane peptide sequence. Also, the intestinal enzyme from rat may be bound to the plasma membrane by hydrophobic interaction (105) and not by the type of attachment proposed by Low (47). The release of alkaline phosphatase by phospholipase C_{II} lends indirect evidence that phosphatidylinositol may

involved in attachment of the enzyme to the plasma membrane but further work will be needed to resolve the issue.

The involvement of calcium and phosphoinositol in the mediation of certain hormones and neurotransmitters has been extensively researched (106,107). Phosphotidylinositol turnover has been shown to be part of the cellular processes taking place upon stimulation of the cell (107) and protein kinases including protein kinase C and cyclic AMP-dependent protein kinases were activated (106,107). If the intimate relationship between the membrane alkaline phosphatase and phosphatidylinositol proposed by Low and his collegues is correct (47), it would mean that alkaline phosphatase may be involved in cell-signal transduction (106), possibly by catalysing a protein phosphatase reaction.

The human liver enzyme can act as a protein phosphatase as evident in our findings and it is evident that alkaline phosphatase can dephosphorylate the plasma membrane phosphoproteins (Fig. 10,11). The physiological substrate(s) of the membrane-bound enzyme may be plasma membrane phosphoproteins and in order to determine the physiological role of this enzyme, the substrate(s) of alkaline phosphatase will have to be identified, requiring the separation, characterization and identification of the liver plasma membrane proteins. The use of purified phosphoprotein substrates, as has been employed in the study of other protein phosphatases (2,18,73), would also be

important for further studies on the protein phosphatase properties of alkaline phosphatase.

CHAPTER FOUR

THE PHOSPHOPROTEINS OF THE HUMAN LIVER PLASMA MEMBRANES

Purified human alkaline phosphatase has been shown to dephosphorylate certain proteins involved in the control of cellular metabolism (9,10). In the previous chapter, we partially characterized the protein phosphatase activity of the endogenous plasma membrane enzyme and presented evidence that identified it as alkaline phosphatase .

Protein kinases as integral plasma membrane proteins have been reported and certain of their membrane protein substrates identified (80-83,110-112). In this regard, two-dimensional polyacrylamide gel electrophoresis has become an important and powerful analytical tool (108,109). In this chapter, we used this technique, and others, to separate and characterize the plasma membrane proteins of human liver that are phosphorylated by endogenous protein kinases.

EXPERIMENTAL PROCEDURES

Gel Electrophoresis

1. One-dimensional. SDS-polyacrylamide gel electrophoresis was performed by a modification of the method of Laemmli (113). The stacking gel composition was 4.78% (w/v) acrylamide and 2.7% (w/v) bis-acrylamide and

the resolving gel was 7% (w/v) acrylamide with 2.7% (w/v) bis-acrylamide. Samples were prepared as described under 'Plasma membrane protein phosphorylation'. Electrophoresis was at 4°C and 30 mA for gel 1.5 mm thick (60 mA for 3 mm gel). The gels were stained either with Coomassie Blue (19) or a silver stain (96). For autoradiography, the gel was soaked in 50% (v/v) methanol, 10% (v/v) glycerol, and 5% (v/v) acetic acid for 15 min and dried under vacuum onto a filter paper. The dried gel was exposed to x-ray film with intensifying screens (114) at -70°C and the exposed film processed in a Kodak X-Omat processor. Radio-labelled proteins separated were also electrophoretically transferred to nitrocellulose sheets (94) and treated as described above for autoradiography.

2. Two-dimensional. Two-dimensional polyacrylamide gel electrophoresis was performed by the method of Rubin and Leonardi (115) in a Bio-Rad Protean II cell. Isoelectric focusing was performed in rod gels (2 mm diameter) cast with 9 M urea, 2% (v/v) Nonidet P-40, 2% (w/v) ampholines (1.6 % pH range 3-10, 0.4 % pH range 5-8), 4% (w/v) acrylamide with 5.4% (w/v) bis-acrylamide. Samples were prepared for electrophoresis as described under 'Plasma membrane protein phosphorylation'. The gels were prefocused at 1.5 mA per gel until voltage reached 400 V. The samples were applied and focusing continued at a constant voltage of 400 V for 18-20 h. Each gel was extruded from the glass

tube and equilibrated in 5 ml (per gel) of a 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, and 625 mM Tris-HCl, pH 6.8 (equilibration buffer). The equilibrated gels could be stored at -20°C until used. Electrophoresis in the second dimension was performed as described (113,115). The slab gel dimension was 160 mm x 30 mm x 1.5 mm for the stacking gel and 160 mm x 120 mm x 1.5 mm for the resolving gel. The equilibrated rod gel is layered onto the stacking gel with tracking dye added [1% (w/v) bromphenol blue in 10% (v/v) glycerol]. Electrophoresis was at 30 mA, constant currunt, until the tracking dye reached to within 1 cm of the bottom of the slab gel. Gels were stained (silver stain) or dried onto filter paper for autoradiography as described above.

For the determination of molecular weights, the following standards were used: myosin (200,000), β-galactosidase (116,200), phosphorylase b (97,400), bovine serum albumin (66,000), and ovalbumin (45,00).

Efficiency of Detergent Solutions in Solubilizing Plasma Membrane Proteins

A suspension of plasma membranes (24 mg protein/ml) was mixed with an equal volume of detergent solution and incubated at room temperature for 5 min. Each mixture (150 l) was centrifuged at 25,000 x g for 10 min (Beckman Airfuge) and the supernatant saved. The samples were

prepared for one-dimensional gel electrophoresis as described under 'Plasma membrane protein phosphorylation'.

Plasma Membrane Protein Phosphorylation

This was carried out in 100 mM Tris-HCl, pH 7.6, 4 mM $MgCl_2$, and 0.4 mM [^{32}P]ATP as described (Chapter 3). Cyclic AMP (5 μM) was present as indicated. For two-dimensional electrophoresis, the membranes were incubated for 15 min at 30°C and the reaction terminated by the addition of 40 μl of solubization buffer [2 % (w/v) SDS, 0.1 M dithiothrietol and 9 M urea], 25 μl of lysis supplement [8% (v/v) Nonidet P-40 and 2% (w/v) ampholines, pH range 3-10] and 100 μl of lysis buffer [2% (v/v) Nonidet P-40, 2% (w/v) ampholines, pH range 3-10 and 9 M urea] per 50 μl of reaction mixture as described (115). The samples were centrifuged at 100,000 x g for 30 min and the supernatant (50 to 70 g protein) applied to each isoelectric focusing gel as described above.

For one-dimensional gel electrophoresis, the protein phosphorylation was terminated by the addition of an equal volume of equilibration buffer, followed by placing the samples in boiling water for 5 min. Concentrated bromphenol blue solution (5 μl) was added to each sample and electrophoresis performed as described above. The running buffer was 0.1% (w/v) SDS, 0.192 M glycine, and 0.025 M Tris-HCl, pH 8.2 (115).

Amino Acid Specificity of the Endogenous Membrane Protein Kinases.

Histones, plasma membranes and a synthetic peptide [poly(GluNa,Tyr), 4:1 Glu:Tyr, 36 kDa (Sigma P-0275)] were phosphorylated by plasma membrane kinases as described (Chapter 3). Following the phosphorylation of the plasma membrane proteins with [^{32}P]ATP, the proteins were hydrolysed in 6 M HCl for 60 min at 100^{o}C. The amino acids were separated and identified by two-dimensional thin layer electrophoresis/chromatography by the method of Hunter and Sefton (97).

With histones and the synthetic peptide as substrate, the plasma membranes were removed by centrifugation. The histones were treated as described above to determined the radio-labelled phosphoamino acid(s). In the case of the synthetic peptide, it was precipitated with trichloroacetic acid [15 % (w/v), final concentration] and the precipitate treated as described for scintillation counting to determine [^{32}P]phosphate incorporation into the tyrosine residues (11).

Plasma Membrane Lipid Phosphorylation

Plasma membranes (5 mg/ml) were incubated in the phosphorylation buffer with or without cyclic AMP as described above, and at the stated times 100 μl of the mixture was removed and the proteins precipitated by the

addition of 100 µl of 25 % (w/v) trichloroacetic acid. A chloroform:methanol mixture (3:1, 2 ml) was added to the protein precipitate and mixed (116). The two phases were allowed to partition under centrifugation (10 min at 5,000 x g) and the lower chloroform layer removed to determine [^{32}P]phosphate content by scintillation counting.

RESULTS

Separation of the plasma membrane phosphoproteins by one-dimension slab gel electrophoresis in the presence of SDS revealed the presence of approximately 25 proteins upon Coomassie staining (PLATE I). In order to enhance detection of the radiolabelled membrane proteins, we applied larger sample volumes (each containing 1 to 1.5 mg protein) by using 3 mm thick slab gels. However as these thicker gels tended to crack on drying, we decided to electrophorectically transfer the [^{32}P]proteins from these gels prior to autoradiography. The time-course of autophosphorylation of the plasma membranes showed the rapid phosphorylation of three proteins with molecular weights (as determined by R_f values) of 74, 45, and 36 kD (PLATE IIb). The autoradiogram of the dried gel also showed these membrane phosphoproteins but with differences in their intensities as compared to the blotted gel (PLATE IIa,b). The electrophoretic transfer of proteins is selective with respect to size and charge (95) and this may account for the

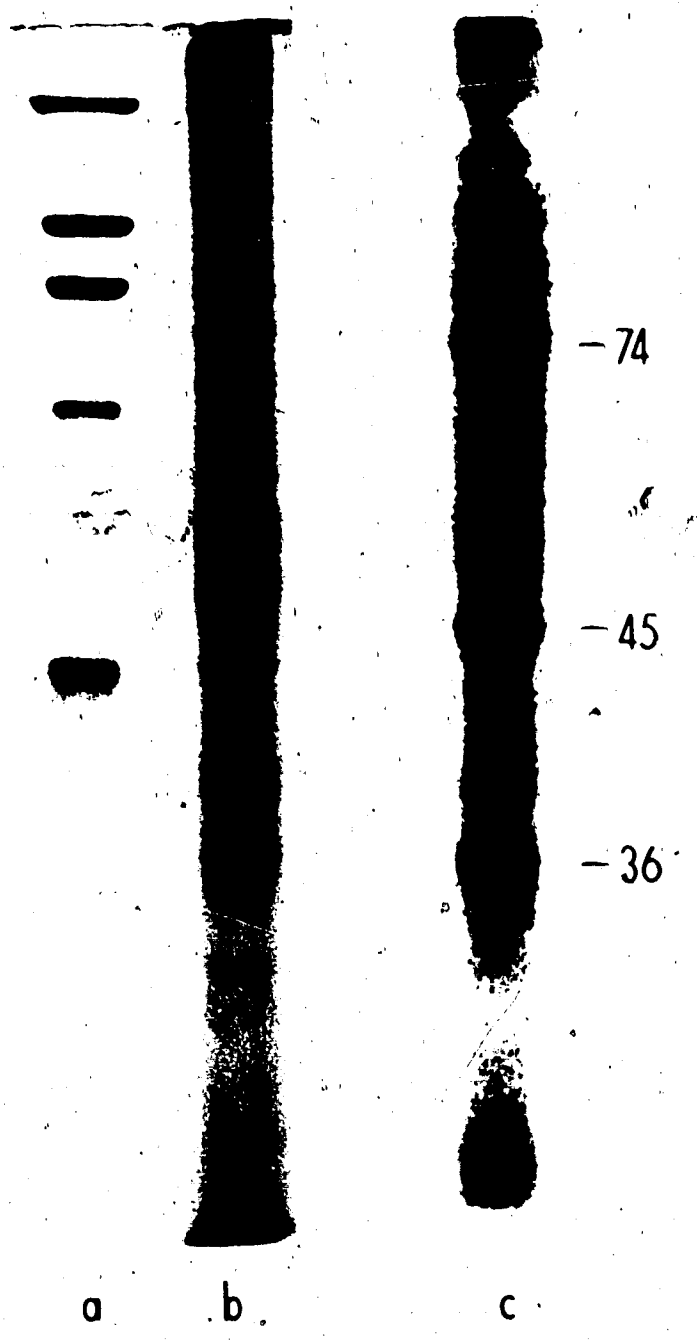

PLATE I SDS-PAGE of Plasma Membrane Proteins. Membrane proteins were autophosphorylated as described in text and resolved on gel electrophoresis in the presence of SDS. Lane a: standard molecular weight markers; lane b: membrane proteins (Coomassie stain); lane c: [^{32}P]phosphate-labelled membrane proteins (autoradiogram).
(note: gel c is not to same scale as gels a,b)

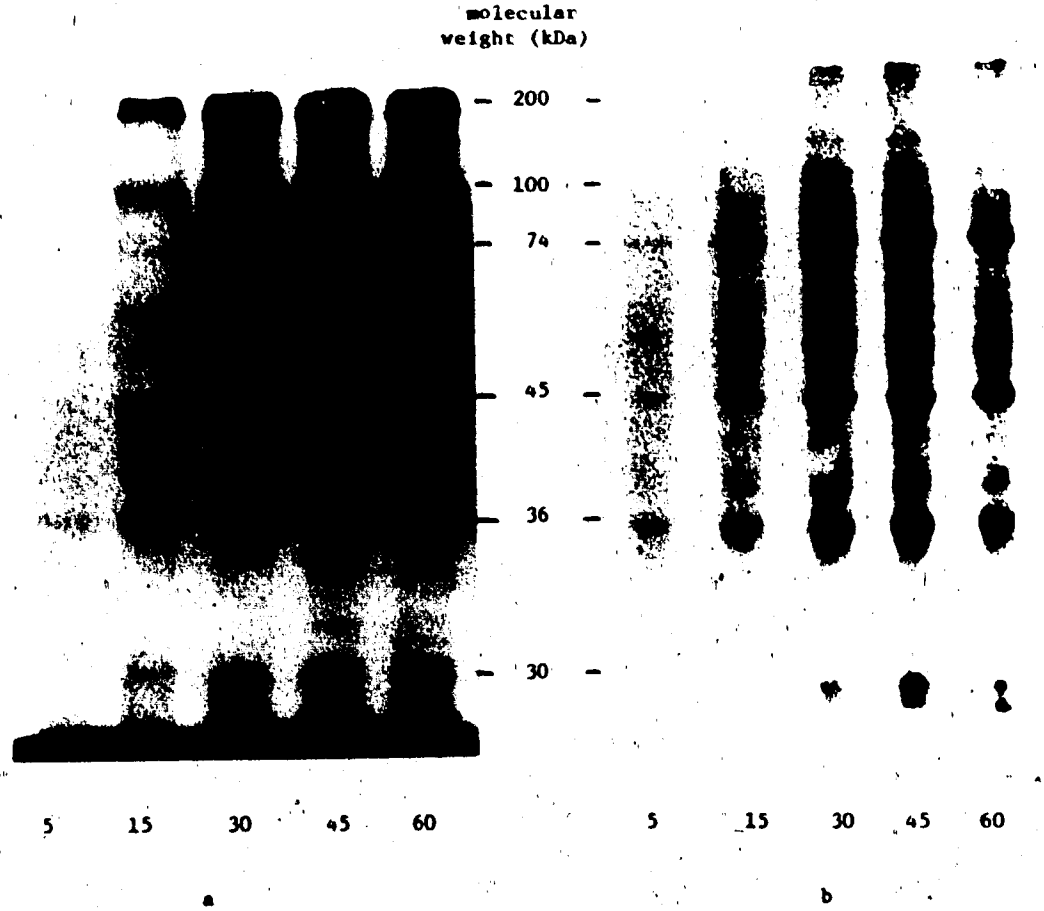

PLATE II **Time Course of Plasma Membrane Protein Auto-Phosphorylation** Membrane proteins were auto-phosphorylated and the reaction terminated at the indicated times by the addition of the SDS solubilization buffer as described in text. The proteins were separated by gel electrophoresis, were either dried onto filter paper (a) or electrophoretically transferred to nitrocellulose paper, and were autoradiographed. The molecular weights of the radio-labelled protein bands were calculated from their R_f values and are as shown.

absence of the 200, 100 and 32 kDa bands from the autoradiogram of the electroblot (PLATE IIb).

In the original two-dimensional gel electrophoresis procedure described by O'Farrell (108), the proteins were solubilized by a non-ionic detergent for the first dimension in order that the detergent would not interfere with the native charge of the proteins. We attempted to follow this protocol but encountered difficulty in solubilizing the plasma membrane proteins with the lysis buffer (TABLE 2). Other modifications of the two-dimensional procedure were tried (109) and the best buffer was one proposed by Rubin and Leonardi (115) with SDS present (PLATE III, TABLE 2). Despite the possible interference of SDS in the first dimension, we believed that the increased resolution of the two-dimensional procedure is the more important consideration. However, the pI's of the membrane proteins determined by this procedure cannot be considered to be those of the native proteins.

Resolution of the membrane proteins by two-dimensional gel electrophoresis and subsequent staining with a silver stain technique revealed the presence of over 80 protein spots (PLATE IV). The autoradiogram of the [^{32}P]phosphoproteins following two-dimensional gel electrophoresis revealed approximately 10 protein spots due to the [^{32}P]phosphate label (PLATE Va). The presence of two radiolabelled protein spots seen on the two-dimensional peptide map when cyclic AMP was not included in the

Lanes: 1 2 3 4 5 6 7

PLATE III <u>Solubilization of Plasma Membrane Proteins by Detergents</u>

Membrane proteins were treated with an equal volume of a detergent solution (see TABLE 2) and the solubilized proteins separated as described in text. The samples were electrophoresed and proteins visualized by Coomassie staining. Lane 1 represents molecular weight standards and 2 to 7 represent samples treated with the respective detergent solutions as detailed in TABLE 2.

TABLE 2. EFFECT OF DETERGENTS ON THE SOLUBILIZATION OF PLASMA MEMBRANE PROTEINS

Lane*	Detergent buffer	Protein solubilized
		% of maximum
2	2% (v/v) Nonidet P-40 in 9.5 M urea, pH 8.0	41
3	2% (v/v) Nonidet P-40 in 50 mM phosphate, pH 7.5	35
4	5 mM K_2CO_3 in 9.5 M urea, pH 9.6	54
5	4 mM CHAPS, aqueous solution	25
6	2% (w/v) SDS in 50 mM phosphate, pH 7.5	100
7	2% (w/v) SDS, 100 mM DTT and 9M urea, pH 8.0	85

*Numbers refer to PLATE III

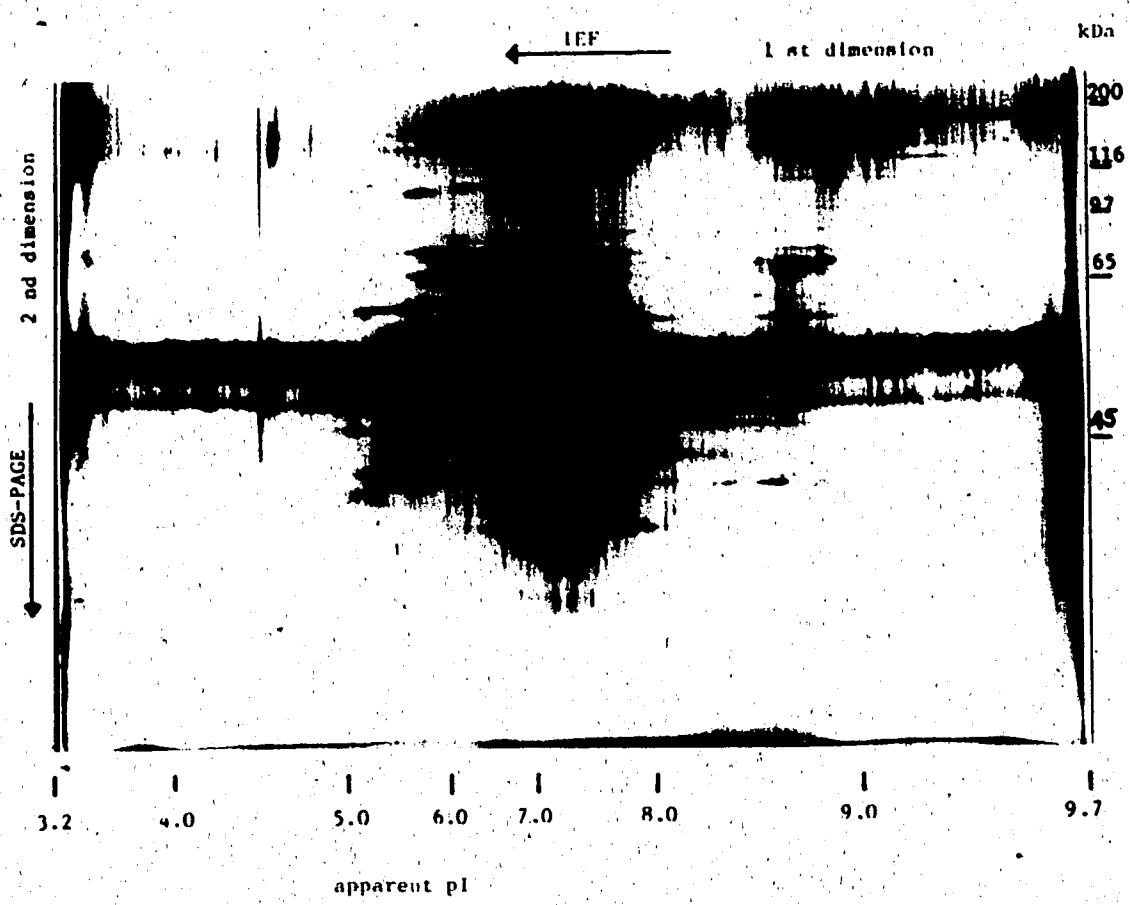

PLATE IV Colour Silver Stain of Membrane Proteins Plasma membrane proteins were separated by two-dimensional gel electrophoresis as described in text. The molecular weights and pI's are as indicated. The origin is at the upper right corner with the anode at bottom for the SDS-PAGE and at left for IEF.

phosphorylation buffer (PLATE Vb) may be due to the endogenous protein kinase(s) being of the cyclic AMP-independent type or due to non enzymatically-catalyzed [^{32}P]phosphate binding (ionic interactions).

The amino acid specificity of the membrane protein kinase(s) was determined by acid hydrolysis of the membrane phosphoproteins and phosphohistones by the method of Hunter and Sefton (97). The membrane proteins and histones were phosphorylated only on serine residues (PLATE VI); no phosphothreonine or phosphotyrosine amino acid was detected. Also, poly(GluNa,Tyr) was not a substrate for the endogenous protein kinase(s) of these plasma membranes

Purified alkaline phosphatase when treated with a cyclic AMP-dependent protein kinase had no significant change in its pNPP hydrolytic activity (TABLE 3). However, we cannot be certain that the alkaline phosphatase was not phosphorylated at other amino acids other than the serine residue at the active site (31,32). Plasma membrane lipids could be autophosphorylated but the phosphorylation mechanism was not dependent on cyclic AMP. We have not identified the membrane lipid substrate(s) or assessed the ability of these phospholipids to serve as a substrate(s) for the membrane-bound alkaline phosphatase. Lipid phosphorylation reached maximum levels after 30 min, representing approximately less than 5% of membrane protein [^{32}P]phosphate incorporation. The autodephosphorylation seen

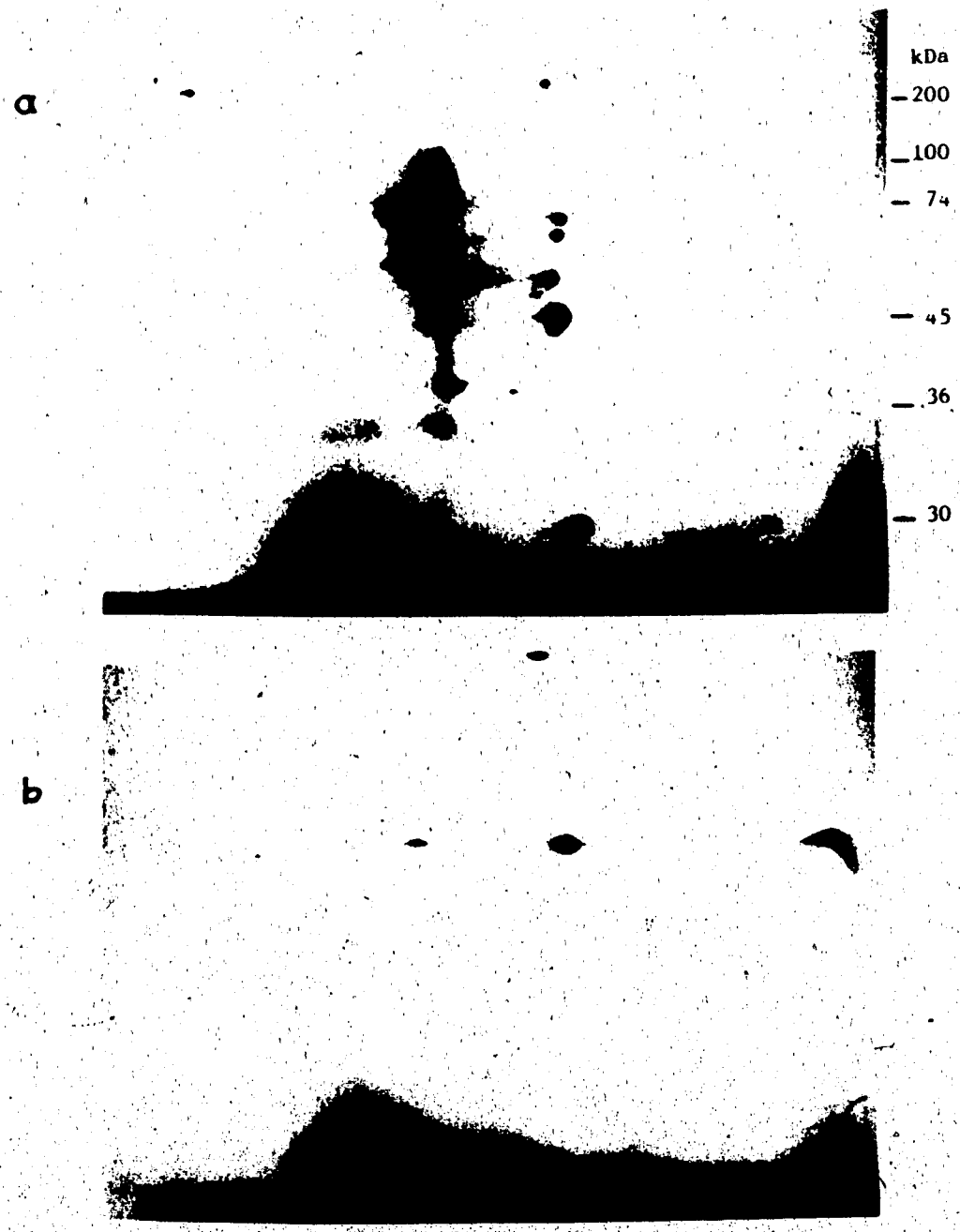

PLATE V **Autoradiograms of Membrane ^{32}P-labelled Proteins**

Plasma membranes were autophosphorylated in the presence (a) or absence (b) of 5 μM cyclic AMP for 15 min and the samples treated and separated by two-dimensional gel electrophoresis as described in text. Following electrophoresis, the gels were dried under vacuum onto filter paper and [^{32}P]-phosphoproteins detected by autoradiography. The gels are oriented as for PLATE IV.

TABLE 3. EFFECT OF CYCLIC AMP ON THE HYDROLYSIS OF pNPP BY MEMBRANE-BOUND AND PURIFIED ALKALINE PHOSPHATASES

Cyclic AMP (μM)	Increase in activity	
	Membrane enzyme	purified enzyme
1	7.3	1.2
5	10.3	7.5
10	10.4	17.8
20	11.4	24.8

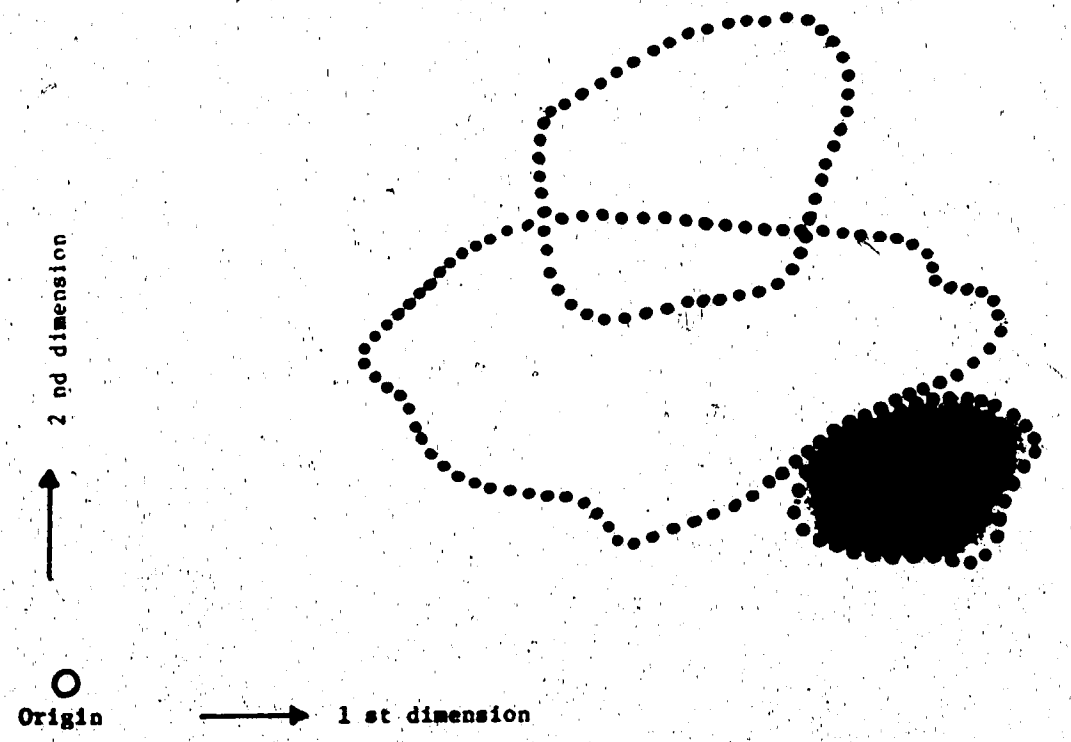

PLATE VI Autoradiogram of [^{32}P]Phosphate-labelled Phosphoamino Acids Plasma membrane proteins were autophosphorylated followed by acid hydrolysis as described in text. The amino acids were separated by a two-dimensional technique and the [^{32}P]phosphoamino acid detected by autoradiography. The dotted lines indicate the position of the phosphoamino acid standards as visualized by ninhydrin. The anode is at the right for the thin layer electrophoresis.

with the membrane phosphoproteins was absent; lipid phosphorylation was not reversible.

DISCUSSION

We found, as part of our efforts to determine the physiological role of human alkaline phosphatases, that the liver plasma membranes exhibited both protein kinase and protein phosphatase activities (Chapter 3), and that membrane alkaline phosphatase was the major protein phosphatase present. The plasma membranes were phosphorylated by endogenous cyclic AMP-dependent protein kinase(s) with at least seven proteins rapidly incorporating the [^{32}P]phosphate label (PLATE II).

Considering the resolving power of two-dimensional gel electrophoresis (108) and the sensitivity of isotopic labelling for the detection of the membrane proteins (108,109), we expected to be able to compare and possibly correlate the radioactive protein bands of the one-dimensional autoradiogram to the spots of the two-dimensional autoradiogram (PLATE I,II,Va). However, the autoradiogram of the phosphoproteins following two-dimensional gel electrophoresis did not reflect the expected results (PLATE Va). The small number of radioactive spots shown by the two-dimensional method with respect to the [^{32}P]phosphoproteins is probably due to lower protein loading capacity of the isoelectric focusing gel, an increased dilution factor, and loss of protein during gel

equilibration between dimensions (109). Another factor may be the dephosphorylation of the [^{32}P]phosphoproteins by the alkaline phosphatase which could still be active even in the presence of SDS (19). The autoradiogram of proteins following two-dimensional gel separation showed the presence of protein spots of similiar molecular weights (PLATE IV). These proteins apparantly have similiar molecular weights but different pI's.

Both plasma membrane protein phosphorylation (via the protein kinases) and alkaline phosphatase activity were activated by cyclic AMP (TABLE 3). The protocol for the autophosphorylation of the plasma membranes also resulted in membrane lipid phosphorylation but this was not cyclic AMP-dependent. Farkas et al (119) has reported the stimulation of lipid phosphorylation in plasma membranes of pig granulocytes by cyclic AMP-dependent protein kinase. Cyclic AMP appears to be involved in many cellular processes and further studies on its role in plasma membrane protein function are needed.

Other workers have reported the phosphorylation of plasma membrane proteins by endogenous protein kinases (80-82,111,112). In human platelets, it was proposed that the granular secretion may involve the phosphorylation of a specific membrane protein (81,82). As seen in the autoradiogram (PLATE IIb), the relative intensities of the radioactivity of the protein bands suggest that the 74, 45 and 36 kDa proteins account for greater than 70% of the

total [^{32}P]phosphate incorporated in the membrane proteins. We have shown that alkaline phosphatase can release greater than 75% of the [^{32}P]phosphate label from membrane phosphoproteins (Chapter 3) and these three prominantly radio-labelled proteins (PLATE II) may be the physiological substrates of the endogenous alkaline phosphatase. The alkaline phosphatases may play an important role in the modulation of the phosphorylation state of the plasma membrane proteins.

CHAPTER FIVE

GENERAL DISCUSSION

The liver is the largest organ in the human body and it performs many diverse functions; it is the site of plasma proteins and carbohydrate synthesis, of lipid metabolism, and of detoxification and hormone metabolism (120). The hepatocytes represent approximately 60% of the gross mass of the liver, and their funtion and that of the entire liver is dependent on the integrity and activity of the plasma membrane and the cellular proteins (120).

The objective of this study was to evaluate the protein phosphatase activity of human alkaline phosphatase as a possible physiological function. The phosphomonoesterase property of human alkaline phosphatase (17) is well characterized but the natural substrate(s) of the enzyme remained unconfirmed.

The regulation of enzyme activity is an important aspect of cellular metabolism and a major mechanism for this in eukaryotes is that of reversible phosphorylation of the key enzymes in a pathway (73). The inter-relationships between cyclic AMP, protein kinases, and protein phosphatases is illustrated in Figure 11 (73). The action of cyclic AMP and the cyclic AMP-dependent protein kinases in the mediation of hormonal and neural signals reflect the importance of reversible phosphorylation in modifying enzyme

activity and ultimately cell function (74). Glycogen metabolism (2), fatty acid synthesis (73), muscle contraction (83), platelet activation (82,83) and neutrophil function (107) are all, in part, controlled by protein phosphorylation.

Cyclic AMP-dependent and -independent protein kinases phosphorylate their protein substrates at specific serine or threonine residues (2,73). Tyrosine-specific protein kinases have also been reported (4-7) but phosphorylation of proteins at tyrosine residues generally tended to occur during cell proliferation, differentiation and transformation (4-7). Also, the tyrosine protein kinases were membrane-bound enzymes as were their putative protein substrates (121). It has been reported that alkaline phosphatase, also a membrane-bound enzyme (18), can act as a phosphotyrosine protein phosphatase (8) due to its apparantly greater affinity for phosphotyrosine with respect to either phosphoserine or phosphothreonine. However, others have argued that alkaline phosphatase is not a tyrosine-specific protein phosphatase in vivo due to the lack of phosphatase inhibition by EDTA in cellular extracts (122); the essential requirement for Zn^{2+} renders alkaline phosphatase sensitive to inhibition by EDTA (Chapter 3): This is not conclusive since other protein phosphatases have metal cation requirement for activity (2). Purified and membrane-bound liver alkaline phosphatase showed no enhanced affinity for a particular phosphoamino acid (18)

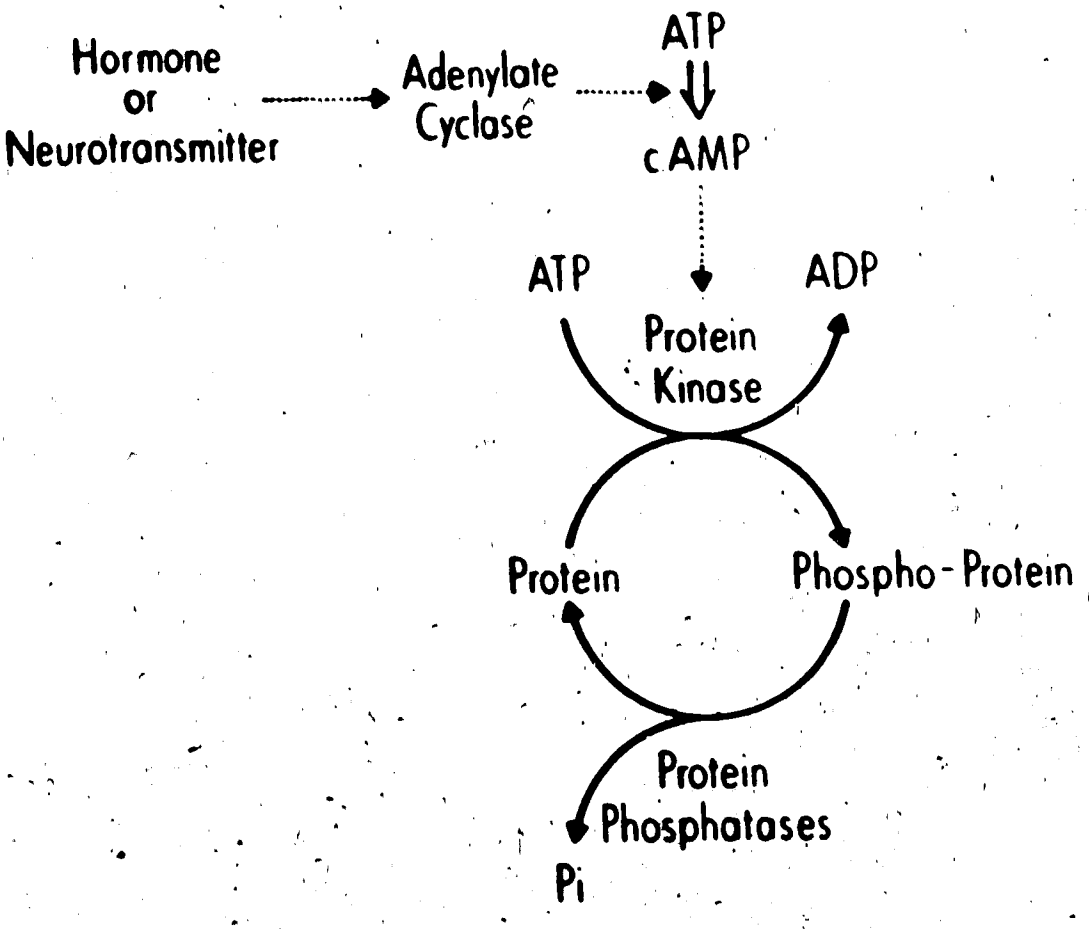

Fig. 12 Schematic of the relationship between cyclic AMP, protein kinases and protein phosphatases (taken from ref. 75).

and the enzyme can act on proteins phosphorylated at either serine, threonine or tyosine residues (8-11). Further work on this aspect of the enzyme will be needed, probably with the use of purified phosphoprotein substrates, in order to resolve the question of the tyrosine phosphatase property of alkaline phosphatase.

It would seem that protein phosphatases essential in a reversible phosphorylation mechanism would also be located in the membrane for controlling the phosphorylation state of the membrane phosphoproteins. This may explain why protein kinases are important components of mammalian plasma membranes (80-82,110-112). It is not understood what role membrane protein phosphorylation may have on the function of the plasma membrane in vivo but it is likely alkaline phosphatase is involved through its ability to catalyze protein dephosphorylation.

The role of alkaline phosphatase in tissue mineralization has been debated at length (36,42,71,72). However, it is difficult to explain why the enzyme is also found in non-calcifying tissues (43,44) unless alkaline phosphatase acts as a protein phosphatase in these tissues. The enzyme, with its ability to hydrolyze both phosphomonoesters and phosphoproteins and to carry out phosphotransferase reactions (11,17), may be expressing different activities depending on its tissue localization.

There were liver plasma membrane proteins phosphorylated by the endogenous protein kinases and

alkaline phosphatase was shown to be capable of acting on these membrane phosphoproteins. Also, it is very likely that most of the membrane phosphoproteins can be the physiological substrates of the membrane alkaline phosphatase. Further characterization of the physical and biochemical properties of these membrane phosphoproteins and their possible purification would be needed. It is hoped that the present study has provided an insight for a better understanding of the physiological importance of the human liver alkaline phosphatase.

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APPENDIX

PUBLICATIONS ARISING FROM THIS THESIS

Chan, J.R.A. and Stinson, R.A. (1985) 'Protein Phosphatase Activity of Human Liver Alkaline Phosphatase' Canadian Congress of Laboratory Medicine, June 22-26; Edmonton, Canada. Abs. #97.

Chan, J.R.A. and Stinson, R.A. (1985) 'Alkaline Phosphatase: A Role in Plasma Membrane Function' Fifth Annual Heritage Medical Research Days, Nov. 21,22; Calgary, Canada. Abs. #47

Hawrylak, K.M., Chan, J.R.A. and Stinson, R.A. (1986) 'Alkaline Phosphatase: Characteristics of a Plasma Membrane Protein' Canadian Biochemical Society: Cellular Structure and Organelles, Apr. 27-May 1; Banff, Canada. Abs. #M15

Chan, J.R.A. and Stinson, R.A. (1986) Dephosphorylation of of Phosphoproteins of Human Liver Plasma Membranes by Endogenous and Purified Liver Alkaline Phosphatases J. Biol. Chem. (in press)

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Dr. D. P. Hollis
The Johns Hopkins University
School of Medicine
Dept. of Physiological Chemistry
Baltimore, Maryland 21205

May 27, 1985

Dear Sir,

I am a graduate student at the University of Alberta in the Dept. of Pathology under the supervision of Dr. R. A. Stinson. In my review of the literature for my thesis, I came across a paper which you and R. S. Zukin have published in the Journal of Biological Chemistry. I am referring to the paper entitled " Role of Metal Ions in Escherichia coli Alkaline phosphatase" (vol. 250, pp 835-842, 1975).

In page 841, there was a figure representing the catalytic metal site of the enzyme. I would like to reproduce the schematic and include it in my thesis. Therefore, I am asking you for your written permission to use this schematic as required by the Faculty of Graduate Studies and Research of the University of Alberta.

I hope I will recieve your answer as soon as possible and I thank you for your kind assisstance.

Yours truely,
John A. Chan

Dear Mr. Chan,

You are welcome to use the figure cited above in your thesis.

Sincerely,

D. P. Hollis.