REGULATION OF MEMBRANE BOUND PROTEIN KINASE ACTIVITY IN *Pisum sativum* L.

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The sun sparkled, the sky was clear, and all the colours he saw seemed to be richer and brighter than he could ever remember. The flowers shone as if they'd been polished, and the tall trees that lined the road shimmered in silvery green.

WELCOME TO EXPECTATIONS, said a carefully lettered sign on a small house at the side of the road....

(The Phantom Tollbooth - Norton Juster)

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ABSTRACT

Membranes isolated from dark grown peas are known to contain protein kinase activity activated by micromolar concentrations of calcium ions. The bud, unexpanded fourth and fifth leaves, shows maximal calcium activation and is the tissue used here. It is shown, by phase partitioning, that a majority of the calcium activated protein kinase activity is located in the plasma membrane. Using acetone to precipitate membrane proteins followed by resuspension in an aqueous buffer (acetone solubilisation) the activity has been solubilised. A calcium activated protein kinase has been partially purified, from this tissue, by a non-denaturing polyacrylamide gel electrophoresis procedure. The enzyme has an approximate molecular weight of 18,000 and shows an ability to autophosphorylate. It is shown that autophosphorylation is activated by calcium plus calmodulin. Apparent positive cooperativity exists for the calcium plus calmodulin activation of autophosphorylation. Data suggests the presence of two calmodulin binding sites and half maximal activation occurs in the micromolar calmodulin range. The autophosphorylation appears to occur on an intra-, as opposed to inter-, molecular basis. Phosphorylation occurs primarily on serine residues that are located in a small peptide fragment(s) produced by tryptic digestion. Upon autophosphorylation the activity of the enzyme towards an exogenous substrate, histone H1, decreases by more than 50%. Autophosphorylation appears to cause aggregation of the enzyme with itself and possibly other proteins. A role for the enzyme as a molecular switch is considered.

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ABBREVIATIONS

cAMP	Cyclic Adenosine Monophosphate.
AMPS	Ammonium Persulphate.
ATBS	Antibody Tris Buffered Saline; 20mM Tris/SO_4^{2-}, 500mM NaCl, pH7.5.
[γ-^{32}P]ATP	[γ-^{32}P] Labelled Adenosine Triphosphate.
Bq	Becquerel.
BSA	Bovine Serum Albumin.
$+Ca^{2+}$	Plus Calcium Buffer; 11mM $MgCl_2$, 55mM HEPES/KOH, 0.45mM EGTA, 0.55 mM $CaCl_2$, pH7.0.
$-Ca^{2+}$	Minus Calcium Buffer; 11mM $MgCl_2$, 55mM HEPES/KOH, 0.45mM EGTA, pH7.0.
^{14}C-FSBA	^{14}C labelled Fluorosuphonylbenzoyl Adenosine.
cpm	Counts per Minute.
Da	Daltons.
dH_2O	Distilled water.
DTT	Dithiothreitol.
EB	Electrode Buffer; 25mM Tris, 192mM Glycine.
EDTA	Ethylenediamine tetra-acetic acid. Disodium salt.
EGF	Epidermal Growth Factor.
EGTA	Ethylene Glycol Bis-(β-Aminoethyl Ether)N,N,N',N' -Tetra acetic Acid.
cGMP	Cyclic Guanosine Monophosphate.
IDP	Inosine Diphosphate.
HEPES	N-2-Hydroxyethyl piperazine-N'-2-ethanesulfonic acid.
HOM	Homogenisation medium; 0.3M Sorbitol, 75mM Tris, 60mM MES, 3mM EDTA, pH7.6.
pI	Isoelectric Point.
+NaCl	Plus NaCl resuspension buffer; 0.5M Sorbitol, 30mM NaCl, 15mM Tris/Maleate, pH7.3. with KOH
-NaCl	Minus NaCl resuspension buffer; 0.5M Sorbitol, 15mM Tris/Maleate, pH7.3. with KOH
MES	2-(N-Morpholino)ethanesulfonic acid.
MW	Molecular Weight.
PAGE	Polyacrylamide Gel Electrophoresis.
βPBD	2-(4'-tert-butylphenyl)-5-(4"-biphenylyl)-1,3,4-oxadiazole.
PBS	Phosphate Buffered Saline; 10mM Na phosphate, 150mM NaCl, pH7.2.
PDGF	Platelet Derived Growth Factor.
RSB	Reuspension Buffer; 0.3M Sorbitol, 6mM Tris, 6mM MES, 0.2mM EDTA, pH7.2.
SBX2	Sample Buffer; 4.7%(w/v) SDS, 10%(v/v) 2-Mercapto ethanol, 20%(w/v) Ficoll or 20%(w/v) Glycerol, 125mM Tris/Cl^-, pH6.8.
SDS	Sodium Dodecyl Sulphate.
SOR	Sorbitol Buffer; 1M Sorbitol, 6mM Tris, 6mM MES, 0.2mM EDTA, pH7.2.
STM	Sorbitol, Tris/maleate resuspension buffer; 0.5M Sorbitol, 3mM Tris/maleate, pH7.3.
TBS	Tris Buffered Saline; 0.9%(w/v) NaCl, 10mM Tris/SO_4^{2-}, pH7.5.
TCA	Trichloroacetic acid.
TCA mix	10%(w/v) TCA, 20mM Na phosphate, 10mM EDTA.
TEMED	N,N,N',N'-Tetramethylethylene-diamine.
TrB	Transfer Buffer; 25mM Tris, 192mM Glycine, pH8.3. For SDS gels 20%(v/v) methanol was included.
Tris	Tris(Hydroxymethyl)-aminomethane.
v/v	Volume for Volume.
w/w	Weight for Weight.
w/v	Weight for Volume.

INTRODUCTION

The aim of this introduction is to outline the background knowledge concerning protein kinases and their control. Specific attention will be given to the role of calcium in plants, both in general terms and as a second messenger. The relevance of the data presented within this thesis is thus made apparent.

Phosphoproteins and Protein Kinases

The presence of phosphate in proteins has been known for over one hundred years (Chock et al. 1980). However, its regulatory potential only became apparent during the 1950's with work on an enzyme involved in glycogen metabolism. Prior to this the only known phosphoproteins were casein, phosvitin and related proteins involved in the feeding of the young or in early embryonic development in animals (Krebs 1983). One enzyme, pepsin, was known to contain phosphate although its function remains unclear (Krebs 1983). Glycogen phosphorylase was known to exist in two forms, active and inactive, and in 1955 Fischer and Krebs showed that these were the result of enzymic phosphorylation-dephosphorylation. Phosphorylation of the enzyme turned it into its active form. Since this time well over fifty enzymes have been shown to undergo such phosphorylation-dephosphorylation, although a regulatory role has not been established in all cases (Krebs 1983). These enzymes include examples from photosynthesis (Bennet 1983), neurone function (Cohen 1982), glycolysis and entry to Krebs cycle (Rao + Randall 1980), contractile processes (Adelstein et al. (1978) and calcium transport (Caroni + Carafoli 1981).

It should be noted that protein phosphorylation-dephosphorylation is not limited to enzymes, many proteins with no enzymic activity also

act as substrates e.g. histones and contractile proteins (Krebs 1983).

The enzymes that catalyze the above phosphorylation reactions are known as protein kinases. The result of their action is the covalent transfer of phosphate from nucleoside triphosphates, primarily ATP, to certain amino acids in substrate proteins (Krebs + Beavo 1979). Such protein phosphorylation represents one form of the many possible post translational modifications of proteins. Whilst protein phosphorylation catalyzed by protein kinases is a reversible reaction (Rabinowitz + Lipmann 1960) phosphoprotein phosphatases also exist. However, work on the latter enzymes has been very limited and consequently little is known of their role (Krebs + Beavo 1979).

Protein Kinase Regulation and Phosphoamino Acid Products

The above section revealed that protein kinases can alter the activity of other enzymes by phosphorylation. By doing so they provide a basic regulatory mechanism for many basic cellular processes in both plants and animals (Greengard 1978). For this to be the case the protein kinases themselves have to be subject to control and the first example of this type to emerge was regulation by cAMP (Krebs *et al.* 1959). Since this time protein kinases regulated by cGMP, double stranded RNA, diacylglycerol(calcium + phospholipid), polypeptide hormones and calcium have been reported (Krebs 1983).

To date, of the above examples, only calcium regulated protein kinases have been unequivocally demonstrated in plants. It is the calcium regulated protein kinases that will be given special attention here.

Soluble calcium activated protein kinases have been partially purified from wheat embryo (Polya + Davies 1982, Polya + Micucci 1984), silver beet leaves (Polya *et al.* 1985a) and soybean

(Putnam-Evans *et al.* 1986). Calcium activated protein kinases associated with plant membranes have also been discovered in pea (Hetherington + Trewavas 1982 + 1984a, Blowers *et al.* 1985), zucchini (Salimath + Marme 1983), ryegrass (Polya *et al.* 1984), corn (Veluthambi + Poovaiah 1984) and tobacco pollen (Polya *et al.* 1985b). However, only for the wheat embryo, soybean and pea protein kinases has the activity been associated with a specific protein of known molecular weight or a defined column fraction. The nature of the calcium activation of these enzymes, and other aspects of cellular calcium are presented in later sections.

Until 1979 protein kinases were considered to use only serine and threonine as substrates, leading to phosphoserine and phosphothreonine respectively. Some work had been carried out on the so called 'acid labile' histone phosphates considered to be phosphohistidine and phospholysine (Chen *et al.* 1974 and Smith *et al.* 1974). Due to the instability of these products in most separation systems little progress has been made (Krebs 1983). The discovery of an ability to phosphorylate tyrosine, giving phosphotyrosine, in Polyoma tumour antigen precipitates (Eckhart *et al.* 1979) opened up a new line in protein kinase research. Polyoma is a DNA virus which transforms animal cells and after infection produces six proteins, three of which are called the tumour antigens (Friedman *et al.* 1979). The presence of the medium tumour antigen and its attachment to the plasma membrane were known to be transformation specific (Ito *et al.* 1977). In the following three years an RNA virus (retrovirus) transforming protein $pp60^{v-src}$ of Rous sarcoma virus (Hunter + Sefton 1980) and the receptors for three animal polypeptide hormones - epidermal growth factor (EGF) (Ushiro + Cohen 1980), platelet derived growth factor (PDGF) (Ek *et al.* 1982) and insulin (Kasuga *et al.* 1982)—were found to

be membrane associated tyrosine specific protein kinases. The polypeptide hormones lead to increased activity of their respective receptors. Many other retroviral products have now been characterised and many, but not all, are membrane associated tyrosine specific protein kinases. Further evidence for a role of tyrosine phosphorylation in cell proliferation appears in the knowledge that lymphoma cell lines express high tyrosine kinase activity (Casnellie et al. 1983) and TPA (12-o-tetradecanoyl-phorbol-13-acetate), a known tumor promoter, stimulates tyrosine phosphorylation in chicken embryo fibroblasts (Bishop et al. 1983). All of the tyrosine kinases described above are in animal systems and to date no tyrosine specific protein kinase has been isolated from plants.

It should be noted that in some circumstances phosphotyrosine may represent as little as 0.05% of the total phosphoamino acid content of an animal cell, rising ten fold upon viral transformation (Sefton et al. 1982). However, levels as high as 50% phosphotyrosine have been reported for red blood cell membranes and platelet cytoskeleton (Tuy et al. 1983), and the significance of this has not been explained.

Autophosphorylation and its Effects on Protein Kinase Activity

Since the protein kinases are themselves protein in nature they are potential substrates for their own activity. Such self phosphorylation is generally termed autophosphorylation and is a common property of many protein kinases (Krebs 1983). In some instances the autophosphorylation is known to alter the properties of the protein kinase and some examples are given below to illustrate this point.

Phosphorylase kinase is usually activated by a phosphorylation brought about by the cAMP dependent protein kinase. However,

autophosphorylation involving sites separate from those of the cAMP dependent protein kinase leads to higher activity when the latter sites become phosphorylated (Wang et al. 1976). The type II cAMP dependent protein kinase consists of regulatory and catalytic subunits which dissociate upon cAMP binding. Autophosphorylation of the regulatory subunit, whilst not affecting cAMP binding (Rosen + Erlichman 1975) retards the reassociation of the subunits (Rangel-Aldao + Rosen 1976). The cGMP dependent protein kinase consists of a single ~75KDa protein that autophosphorylates, although no regulatory role for this is known (De Jonge + Rosen 1977). Likewise casein kinase II autophosphorylates, although no regulatory role has been established (Geahlen et al. 1986). Glycogen synthase kinase autophosphorylates and reduces its own activity (Yamauchi + Fujisawa). The type II calcium dependent protein kinase autophosphorylates and reduces its activity towards other substrates by ~65%. However, the activity that remains is then totally independent of calcium (Miller + Kennedy 1986). The autophosphorylation of this enzyme is sufficient to alter its electrophoretic mobility even in SDS polyacrylamide gels (Yamauchi + Fujisawa 1985).

Finally, all of the tyrosine kinases mentioned above exhibit autophosphorylation on tyrosine residues. For one of them, the insulin receptor, it has been shown that autophosphorylation is necessary for activation of the kinase activity directed towards other substrates (Rosen et al. 1983).

Of the calcium activated protein kinases identified in plants (see above) only one appears to exhibit an autophosphorylating activity (Blowers et al. 1985 plus results presented here).

Substrates for Calcium Activated Protein Kinases in Plants

This remains one of the great unknowns. Whilst autophosphorylation of the protein kinase of Blowers et al. (1985 plus results presented here) shows that this enzyme acts as its own substrate, in only one other case has a substrate been identified. Refeno et al. (1982) reported that the enzyme quinate:NAD oxidoreductase, catalyzing the reversible oxidation of quinate, was regulated by phosphorylation-dephosphorylation. However, the calcium activated protein kinase involved requires millimolar calcium ions. As will be outlined below, this concentration of calcium ions is one that would not be encountered in the cytoplasm of a cell.

A point which has been avoided thus far is the precise role of calcium in the activation of enzymes, specifically protein kinases. Before considering the protein which senses changes in intracellular calcium, calmodulin, an introduction to calcium as a plant nutrient and ion will be given.

Calcium at the Whole Plant and Cellular Levels

The requirement of calcium as a plant nutrient has been recognised for over one hundred years (Kirkby + Pilbeam 1984) and several physiological disorders (e.g. bitter pit of apples, black heart of celery and blossom end rot of tomatoes) have been associated with localised deficiencies of calcium within the plant (Kirkby + Pilbeam 1984). Calcium enters the plant through the root, primarily the tip, and travels in the apoplastic spaces to the stele (Roux + Slocum 1982). In more mature regions of the root some symplastic transport across the endodermis is required (Pitman 1977). Once calcium enters the xylem its transport is unidirectional, from the

roots to the meristematic zones and young tissues (Bangerth 1979).

In the plant tissues the bulk of the calcium is found in the apoplastic compartment, complexed with the cell wall and the plasma membrane (Clarkson + Hanson 1980). In the latter case the calcium ions act to stabilize the membranes, by crosslinking between phosphate and carboxyl groups of phospholipids and membrane proteins (Legge 1982). In the cytoplasm a major portion of the calcium present is bound to internal membranes and ligands e.g ATP, citrate and oxalate (Campbell 1983). The concentration of free, uncomplexed, calcium ions external to the cell is in the millimolar range, whilst in the cytoplasm the value is considerably lower. Many attempts have been made to measure the free calcium ion concentration in plant cells. Due to the presence of a cell wall and esterases on the cell surface many of the techniques used in animal cells are not suitable. However, two reports have appeared in the literature (Williamson + Ashley 1982 and Gilroy et al. 1986), both indicating that cytoplasmic free calcium is in the sub-micromolar range. It is necessary for the cell to maintain a low concentration of intracellular calcium. If the cytoplasmic calcium concentration rose to the level found outside the cell then insoluble precipitates with inorganic phosphate would form, bringing phosphate based energy metabolism to a halt (Hepler + Wayne 1985). To maintain the cytoplasmic calcium concentration at such a low level the ion has to be actively pumped out of this compartment (Hepler + Wayne 1985). It is generally assumed that the control of cytoplasmic calcium in plant cells is achieved as in animal cells, via extrusion out of the cell and sequestration in organelles. A variety of calcium pumps have been identified in numerous membrane fractions including the plasma membrane (Dieter + Marme 1980a), endoplasmic reticulum (Buckhout 1983), tonoplast (Gross 1982) and mitochondria

(Dieter + Marme 1980b). However, the role of the mitochondria, and chloroplasts, is probably minor compared to that of the vacuole (Thomas 1986).

Calcium as an Ion and Second Messenger

It is now generally accepted that calcium acts as a second messenger in plant cells i.e. it acts to translate primary signals such as phytochrome transformation and plant growth substances into a final response. However, knowledge at present is at an early stage when compared to that of animal systems (see Campbell 1983).

The question arises - why calcium? To answer this the properties of calcium as an ion, when compared to other candidates, must be considered. As outlined above, the cell requires the maintenance of a low (sub-micromolar) cytoplasmic calcium concentration. The result is a steep gradient of calcium across membranes such as the tonoplast and plasma membrane. Calcium is thus poised to 'flow down' its electrochemical gradient. The concentration of magnesium and sodium ions in the cytoplasm is millimolar and potassium is one tenth molar. The concentration of calcium ions in the cytoplasm can increase by 100 fold without disturbing the ionic milieu of the cell, but even a small fold increase in magnesium, sodium or potassium would disturb the osmotic and charge balance of the cell (Hepler + Wayne 1985). Large amounts of energy would also be expended in returning the cell to its resting state.

Properties of the calcium ion itself that make it suitable to act as a second messenger are its low free energy of hydration and variable coordination number (Haug + Weis 1986). The former allows more rapid exchange of water in binding to target sites when compared to, say, magnesium (Hepler + Wayne 1985). The latter allows

flexibiliy in fitting to binding sites when compared to the fixed coordination geometry of, say, magnesium (Haug + Weis 1986).

Calcium Entry Into the Cytoplasm

Having described above the mechanisms involved in maintaining a low cytoplasmic calcium concentration, a rise, as produced by a primary stimulus, must now be considered. There are four possible routes for calcium to cross a membrane (Campbell 1983); i) through specific channels, ii) permeation around proteins in the membrane, iii) through neutral cal cium permeases and iv) neutral calcium movement through the lipid bilayer. Artificial membranes composed only of phospholipids, with no proteins, are highly impermeable to ions (Dormer et al. 1978). Whilst the insertion of proteins into the bilayer increases the ionic permeability by several orders of magnitude, the permeability to calcium ions is still relatively low (Campbell 1983). Of the above mentioned routes for calcium to cross a membrane only specific channels allow the rapid transfer of large numbers of ions. Calcium channels are known to exist in animal cells and are assumed to exist in plants. Two successful reports, using drugs designed to bind to animal calcium channels, on plant tissues have appeared in the literature (Hetherington + Trewavas 1984b and Andrejauskas et al. 1985). The latter report indicates a major portion of the channels to be located in the plasma membrane, with some in the endoplasmic reticulum or tonoplast.

As outlined by Thomas (1986) a rise in the cytoplasmic concentration of free calcium ions can arise from a number of sources. As well as a release of chelated calcium, entry into the cytoplasm from membrane bound intracellular stores (see above) is also possible. Due to the low diffusion rate of calcium through the cytoplasm large

concentration gradients can occur over relatively small distances, thus the cytoplasmic free calcium concentration can vary in space as well as time.

Calmodulin - a Calcium Binding Protein

The first substantial information concerning the the molecular mechanisms by which calcium signals act came from studies of the control of muscle cell contraction. The discovery of troponin C, a calcium dependent regulatory protein, represented the first example of any such protein. However, it is specifically located in striated muscle tissue (Klee + Vanaman 1982). Calmodulin was first detected as a protein factor that stimulated the activity of brain cyclic nucleotide phosphodiesterase (Cheung 1970). In 1973 Teo and Wang demonstrated that this activity was a consequence of calcium binding to calmodulin.

Calmodulin is now recognised to be a member of a family of calcium binding proteins including troponin C, parvalbumin, intestinal calcium binding protein and S-100 (Babu et al. 1985). All of these proteins, except calmodulin, are restricted to vertebrates and even a small number of tissues or cells (Babu et al. 1985). Whilst ubiquitous in eukaryotes no calmodulin has been found in any prokaryote so far examined, although bacteria do contain small acidic calcium binding proteins (Klee + Vanaman 1982).

Calmodulin is a small heat stable and very acidic protein (pI ~3.4) that shows a remarkably conserved sequence in evolution (Roberts et al. 1986). It consists of a single polypeptide chain of 148 amino acids and contains four calcium binding domains (Klee + Vanaman 1982). At the resting, sub-micromolar, cytoplasmic free calcium ion concentration calmodulin is inactive and has no calcium bound to it.

The affinity constants of the binding sites for calcium are in the micromolar range. An increase in the cytoplasmic free calcium ion concentration by 100 fold (to, say, ~10 micromolar) is sufficient to saturate all four binding sites and produce a conformational change in the calmodulin molecule (Klee + Vanaman 1982). This conformational change leads to an increase in the affinity of calmodulin for its target proteins. The binding of calmodulin to target proteins is necessarily calcium dependent and such an association may both amplify and lengthen the initial stimulus (Moore + Dedman 1982). Half maximal activation of enzymes by calmodulin, a measure of the affinity of binding, are often in the low nanomolar range e.g. for phosphodiesterase and NAD kinase (Klee + Vanaman 1982). However, values approaching micromolar have been obtained for other enzymes (e.g. Polya + Micucci 1984 and Landt et al. 1982). It is the conformational change upon calcium binding that also allows the binding of specific drugs to calmodulin. Trifluoperazine (TFP) is one such drug which interacts with calmodulin and prevents its binding to target proteins (Klee + Vanaman 1982).

The three dimensional structure of calmodulin has also been resolved (Babu et al. 1985) showing that it consists of two globular lobes, each containing two calcium binding domains, separated by an exposed alpha helix region.

In cells calmodulin is distributed between the soluble and particulate fractions. Membrane associated calmodulin may be very tightly bound, requiring treatments that destroy the membrane structure for its removal (Kakiuchi et al. 1978). Translocation of calmodulin from one compartment of the cell to another may represent a mechanism for the control of calmodulin action (Klee + Vanaman 1982). Gnegy et al. (1977) reported release of calmodulin from brain synaptic

membranes upon phosphorylation of proteins by the cAMP dependent protein kinase.

Amplitude or Sensitivity Modulation?

As outlined by Hepler and Wayne (1985), two types of control involving calcium as a second messenger are possible. The two types of control have been termed 'amplitude' and 'sensitivity' modulation (Rasmussen 1983).

Amplitude modulation depends on an increase in the cytosolic free calcium ion concentration. Such an increase would activate calmodulin, or another calcium binding protein, and its subsequent binding to a target protein would give the response. However, due to the cytotoxicity of prolonged exposure to raised levels of cytosolic calcium, such control is not suitable for sustained responses. The calcium is actively pumped out of the cytoplasm and the cell returns to a resting state as the calcium binding protein becomes inactivated.

Sensitivity modulation has been identified in animal systems and two examples will serve to illustrate this form of control. A protein kinase, protein kinase C, has been discovered in animal cells and shows a dependence on both calcium and phospholipid (Takai et al. 1979). In response to many agonists there is an increase in phospholipase C activity and a subsequent release of diacylglycerol from the plasma membrane. Diacylglycerol binds to protein kinase C and increases its affinity for calcium such that its activity remains even at resting concentrations of cytoplasmic free calcium.

The other form of sensitivity modulation involves covalent modification, and can be illustrated by the brain type II calcium and calmodulin dependent protein kinase of Miller and Kennedy (1986), discussed in an earlier section. The enzyme is activated by an

increase in cytoplasmic free calcium ion concentration and binding of activated calmodulin. Its ability to autophosphorylate leads to a ~65% decrease in activity, but this residual amount is independent of calcium and calmodulin. The initial response is thus extended without the need for prolonged periods of elevated cytosolic free calcium.

Summary

The scene is set. The plant cell appears to contain all of the necessary components for stimulus-response coupling via an increase in cytosolic free calcium. Calcium channels are thought to exist, allowing the rapid influx of calcium into the cytoplasm. Calcium pumps are present, and their action would return the cell to a resting state. The calcium binding protein calmodulin is present in plants, as are calcium and calmodulin dependent enzymes. The calcium signal can thus be translated into precise metabolic changes within the cell.

One family of enzymes, the protein kinases, are in some cases regulated by calcium and calmodulin and form an ideal starting point for amplification and diversification of response (Shacter-Noiman et al. 1983). Clearly, the isolation and characterisation of calcium and calmodulin regulated protein kinases from plants will lead to a greater understanding of the sequence of events from primary stimulus to final response. However, without a precisely defined primary stimulus and a complete understanding of the whole cell, reactions in vitro remain isolated.

MATERIAL AND METHODS

MATERIAL

Plant Material

Peas (*Pisum sativum* L. cv. Feltham First) were obtained from W.K.McNeir, Edinburgh.

Radiochemicals

[γ-^{32}P]Adenosine triphosphate ([γ-^{32}P]ATP) was obtained from Amersham International plc (Amersham, Bucks., England) as the stabilised aqueous triethylammonium salt. Specific activity either >185TBq $mmol^{-1}$ or 111 TBq $mmol^{-1}$. Stored at -20°C.

[8-Adenosine-^{14}C]5'-Fluorosulphonylbenzoyladenosine (^{14}C-5'-FSBA) was obtained from New England Nuclear (Du Pont, Herts., England) in 95%(v/v) ethanol. Specific activity 1.6 GBq $mmol^{-1}$. Stored at -20°C.

Film

X-ray film Cronex-4, Intensifying screens Cronex Xtralife-lightening plus Du Pont (Herts., England.)

Photographs taken using Technical Pan 2415 or, for line figures, Kodalith.

Chemicals and Media

Bovine calmodulin - Calbiochem (Bishops Stortford, U.K.)

All other chemicals were obtained as analytical grade reagents from either Sigma London Chemical Co. (Poole, Dorset, U.K.) or BDH Chemical Co. (Poole, Dorset, U.K.)

Cellulose discs (diameter 2.1cm), Phosphocellulose paper-P81 and 3MM paper - Whatman (Maidstone, Kent, England.)

Nitrocellulose (pore size 0.2μM) - Schleicher and Schüll (Dassel, FRG.)

Cellulose thin layer plates (plastic backed 100µm) - Merck + Co. Inc. (Rahway, N.J., USA.)

Sephadex G100 (standard grade) - Pharmacia Fine Chemicals (Uppsala, Sweden.)

Horse radish peroxidase Goat-anti-Mouse conjugated antibody kit - Bio-Rad Laboratories (Richmond, Cal., USA.)

Centrifuges

Superspeed-65 ultracentrifuge and Micro-Centaur, MSE scientific instrumemts (Manor Royal, Crawley, England). Sorvall RC-5B, Du Pont Company (Biomedical Products Division, Newton, Con., USA.)

Scintillation Counter

Kontron - Intertechnique, France.

METHODS

All procedures, unless otherwise stated, were carried out at approximately 4°C.

Growing Conditions

Peas were imbibed overnight in running tap water to aid germination. The imbibed peas were grown in moist vermiculite in the dark at 20-22°C for 12 days.

Harvesting of Plant Material

Plant material used was as described by Hetherington and Trewavas (1984a) i.e. the bud which contains the unexpanded fourth and fifth leaves. Buds were harvested and either placed on ice for direct processing or frozen in liquid nitrogen and stored at -80°C.

Membrane Isolation

Tissue was homogenised in two to three volumes per gram fresh weight of HOM. Homogenates were squeezed through four layers of muslin and the resulting suspension centrifuged at 17,000 g for 10 minutes in a Sorvall SS34 using a Sorvall RC-5B centrifuge. The

resulting supernatant was then centrifuged at 48,000 g for 30 minutes as above. Pellets were resuspended in RSB using a glass-teflon homogeniser and 6ml aliqouts layered onto 6.5ml sorbitol buffer (SOR) to form a discontinuous gradient. Centrifugation was performed using an MSE 6X14ml Titanium swing out head in an MSE Superspeed-65 ultracentrifuge at 150,000 g for 1 hour 30 minutes. The washed membrane pellets were resuspended in resuspension buffer (RSB), or plus NaCl resuspension buffer (+NaCl) for phase partitioning, and are referred to as 'total' membrane.

Acetone Precipitation of Membranes and Resuspension (Acetone Solubilisation)

The method used is a modification of that of Venis (1977). After resuspension the membrane preparations were added dropwise to twenty volumes of gently swirling ice cold acetone and left to stand for 30 minutes. A pellet was produced by centrifugation for 5 minutes at 3,000 g in a Sorvall SS-34 rotor using a Sorvall RC-5B centrifuge. The pellet was resuspended in RSB, RSB +20%(v/v) glycerol or PBS in a motorised 1ml glass-teflon homogeniser for 3 minutes at 50revs. minute^{-1}. In some cases the preparation was left to stand on ice for 15 minutes. Centrifugation was at 40,000 g for 30 minutes in a 10X10ml aluminium MSE rotor in an MSE Superspeed-65 ultracentrifuge. The supernatant was recovered and is referred to as the acetone solubulised membrane fraction.

Phase Partitioning of Membranes

The methods used were those of Yoshida et al. (1983). 'Total' membrane fractions were resuspended in +NaCl and 0.5ml aliquots added to produce 4 gram phases with a final composition of 5.6% (w/w) each of polyethylene glycol (approximate molecular weight 3,350 Da) and dextran (average molecular weight 472,000 Da) in +NaCl buffer. Phases

were thoroughly mixed by 30 inversions and left to settle for 10 to 30 minutes at 0°C. Brief centrifugation in a bench centrifuge was used to aid phase separation. Upper phases were separated from lower with a Pasteur pipette, leaving the interface with the lower phase. Both upper and lower phases were then diluted by the addition of 6ml minus NaCl buffer (-NaCl) and thorough mixing. Centrifugation was performed in an MSE 10X10ml aluminium rotor using an MSE Superspeed-65 ultracentrifuge at 150,000 g for 1 hour 30 minutes. The resulting sorbitol Tris/maleate (STM) resuspended upper phase pellet is referred to as the 'plasma membrane enriched' fraction, and that for the lower phase as the 'residual' membrane.

Isopycnic Sucrose Density Gradient Centrifugation

Linear sucrose gradients (13ml) containing 15 to 50%(w/w) sucrose in 10mM Tris/maleate, 1mM EDTA, pH7.3 were constructed using a gradient maker (Hoeffer Scientific Instruments, San Francisco, Cal., USA.). Membrane preparations (0.5ml) were loaded onto the gradients in the above buffer with 10%(w/w) sucrose. Centrifugation was performed at 96,000 g for 13 hours at 4°C using an MSE 6X14 Titanium swing out head in an MSE Superspeed-65 ultracentrifuge. Fractions were collected from the bottom of the gradient and sucrose content determined using a refractometer (Bellingham + Stanley, London, England).

Membrane Marker Assays

NADH Cytochrome c reductase assays were performed as described by Lord (1983). Reactions were initiated by addition of 100µl of a suitably diluted membrane preparation to 0.9ml of assay buffer leading to a final composition of; 0.2mM NADH, 20µM Cytochrome c (Horse heart -Sigma C-2506), and 20mM KCN in 20mM sodium phosphate buffer pH7.2.

Reduction of cytochrome c was followed as an absorbance increase at 550nm in a Pye Unicam SP8-100 ultraviolet spectrophotometer.

Latent inosine diphosphatase (IDPase) assays were performed as described by Green (1983). Membrane preparations were stored at 4°C for 3 days prior to assay. Reactions were initiated by addition of 25µl of a suitably diluted membrane preparation to 225µl of assay buffer containing 3mM IDP as the sodium salt, 1mM $MgCl_2$ and 30mM Tris/HCl pH7.5. After incubation for 30 minutes at 20°C the reactions were terminated by the addition of 250µl 10%(w/v) trichloroacetic acid (TCA) and placed on ice for 10 minutes. Precipitated protein was pelleted by centrifugation at 11,600 g in an MSE Micro-Centaur centrifuge for 10 minutes. Supernatants were removed and released phosphate determined by the method of Taussky + Shorr i.e the addition of 1ml 1% (w/v) ammonium molybdate in 2N H_2SO_4 followed by 0.2ml Fiske+Subbarow reducer. After 35 minutes at room temperature absorbance was determined at 660nm in a Pye Unicam SP8-100 ultraviolet spectrophotometer.

Cytochrome c oxidase assays were performed as described by Moore and Proudlove (1983). Membrane preparations were resuspended in STM and 135µl of a suitably diluted fraction added to 15µl of 40mgml^{-1} digitonin. After incubation for 1 minute at room temperature 750µl of 0.1M sodium phosphate buffer (pH7.0) and 100µl of reduced cytochrome c were added. The cytochrome c had been previously reduced with sodium dithionite until $A_{550/515}$ approached 9 to 10. Cytochrome c oxidase activity was taken as the initial rate of decrease in absorbance at 550nm.

Protein Kinase Assays - Non-site Specific

Two such methods were employed. A cellulose disc method for membrane preparations and a phosphocellulose strip method for all

solubilised and subsequent preparations. [γ-^{32}P]ATP generally added at a minimum of 1µl per 50µl total assay volume, final concentration ~0·1µM.

For the cellulose disc method the basic assay consisted of equal volumes of minus/plus calcium buffer at double strength (-/+Ca^{2+}X2) and the membrane preparation in question. [γ-^{32}P]ATP was added and at the appropriate times aliquots removed to Whatman 3MM cellulose discs which had been pre-treated with 0.1ml TCA mix. All discs, including 'no enzyme' blanks, were stored overnight in a large volume of TCA mix. After draining off the TCA mix the discs were soaked in 100% (w/v) TCA for 20 minutes and then transferred to boiling TCA mix for 15 minutes. After cooling for 30 minutes the discs were washed in acetone containing 2%(v/v) 1M HCl and air dried. Incorporated phosphate was estimated by Cerenkov counting of each disc in 5ml dH_2O.

The phosphocellulose method was performed exactly as described by Roskoski (1983). The basic assay, as described above, was aliquoted at the appropriate times to 1X2cm Whatman P81 phosphocellulose strips and immediately added to 75mM phosphoric acid at 10ml/strip. 'No enzyme' blanks were added last since the desorption of ATP is time dependent. Strips were washed in three further changes of phosphoric acid and air dried. Incorporated phosphate was estimated by Cerenkov counting of each strip in 5ml dH_2O.

Tyrosine Specific Protein Kinase Assays

The method of Braun et al.(1983) was used with both tyramine and angiotensin II as exogenous substrates. Preparations were assayed in a buffer of final composition:- 20mM HEPES, 10mM DTT, 10mM $MgCl_2$ 1mgml^{-1} BSA, 100µM $CaCl_2$ or 200µM EGTA, pH7.4 and with or without the exogenous substrate. [γ-^{32}P]ATP was added and at the appropriate times 25µl aliquots were removed and added to 100µl 25% (w/v) TCA. After standing on ice for 30 minutes, precipitated protein was pelleted by

centrifugation at 11,600 g in an MSE Micro-Centaur centrifuge for 10 minutes. 100µl of 2M HCl was then added to 100µl of the supernatants and heated at 100°C for 20 minutes in a Tecam DB-3 dri-block (Techne, Cambridge, England) to hydrolyze residual [γ-^{32}P]ATP. After cooling, 4ml 1.25M perchloric acid, 4ml 2-methyl propan-1-ol:benzene 1:1 and 1ml 5%(w/v) ammonium molybdate were added and thoroughly mixed. After aspiration of the organic phase the aqueous phase was extracted twice with 2ml dH_2O saturated 2-methylpropan-1-ol. The organic phase was again aspirated and a final extraction performed with 2ml diethyl ether. Phosphate was estimated by Cerenkov counting of the aqueous phase, which should contain any phosphorylated exogenous substrates.

Gel Electrophoresis

Sodium-dodecyl-sulphate (SDS) polyacrlamide gel electrophoresis (PAGE) was performed using a discontinuous gel buffer system as follows:- Stacking gel containing 5%(w/v) acrylamide, 0.13%(w/v) bis-acrylamide, 125mM Tris/HCl (pH6.8), 0.1%(w/v) SDS and 0.03%(w/v) AMPS plus 0.1%(v/v) TEMED to polymerize. Resolving gel containing 12%(w/v) acrylamide, 0.32%(w/v) bis-acrylamide, 375mM Tris/HCl (pH8.8), 0.1%(w/v) SDS and 0.015%(w/v) AMPS plus 0.05%(v/v) TEMED to polymerize. Electrode buffer as EB with 0.1%(w/v) SDS. Dimensions of resolving gels were either 16X16X0.1cm or 8X5X0.15cm, the latter being a Mighty Small vertical slab gel unit (Hoeffer Scientific Instruments, San Francisco, Cal., USA.). Samples for SDS-PAGE were added to an equal volume of sample buffer (SBX2) and bromophenol blue added at 0.014%(w/v). After heating in a Tecam DB-3 dri-block (Techne, Cambridge, England.) for 10 minutes at 100°C the samples were loaded into the gel slots. Running conditions were 300V, 20mA (constant current) for the large gels and 150V, 18mA (constant current) for the Mighty Small. BSA (68KDa), Catalase (60KDa), Aldolase (40KDa),

Carbonic anhydrase (29KDa), Soybean trypsin inhibitor (21KDa), Myoglobin (17.2KDa) and Lysozyme (14.3KDa) were used as molecular weight markers. Both gel systems were run at room temperature.

PAGE under non-denaturing conditions was carried out using a discontinuous gel buffer system as follows:- Resolving gel containing 7%(w/v) acrylamide, 0.19%(w/v) bis-acrylamide, 12.5%(v/v) glycerol, 375mM Tris/HCl (pH8.3) and 0.017%(w/v) AMPS plus 0.07%(v/v) TEMED to polymerize. Electrode buffer as EB. No stacking gel was employed and the gel dimemsions were 20X16X0.1cm. Samples for non-denaturing PAGE were labelled in the presence of 10%(v/v) glycerol and loaded onto the gel. Separation was performed at 4°C for approximately 16 hours at a constant 150V (7.5Vcm^{-1}). A few drops of 0.2%(w/v) bromophenol blue were added to the upper gel tank to act as a tracking dye.

Renaturation of SDS Polyacrylamide Gels

The first method was based on that of Kuret and Schulman (1985). SDS polyacrylamide gels were transferred to 25%(v/v) propan-2-ol, 10%(v/v) ethanoic acid for 20 hours with three changes. This was followed by two half hour washes in dH_2O and a half hour wash in $+Ca^{2+}$. After another half hour wash in $+Ca^{2+}$ with bovine calmodulin at 10μg per μl [γ-^{32}P]ATP was added and the gel incubated for 4 hours. The gel was then washed in 5%(w/v) TCA, 1%(w/v) sodium pyrophosphate until all background activity was removed.

The second method used was a modification of that described by Geahlen et al. (1983). After separation of proteins by SDS-PAGE the gels were washed for 4 hours at room temperature in three 100ml changes of 50mM HEPES (pH7.4) to remove SDS. Following this the gels were washed in $+Ca^{2+}$ for ~1 hour at 4°C and then for a further hour in $+Ca^{2+}$ with 100μg per ml of bovine calmodulin and ~1.5 MBq [γ-^{32}P]ATP. To remove un-incorporated ATP the gels were rinsed briefly in dH_2O and

then overnight in 200ml of 50mM HEPES (pH7.4) with 10g of 'HEPES washed' Dowex 1X8-50 chloride form anion exchange resin. After a final wash in 200ml 10%(w/v) TCA, 1%(w/v) sodium pyrophosphate for 1 hour the gels were rinsed with dH_2O and stained with Coomassie Brilliant Blue.

Equilibration of Non-denaturing Gel Slices for SDS-PAGE

Slices from 'wet' non-denaturing gel slices were eqilibrated in 0.5ml of equilibration buffer (10%(v/v) glycerol, 5%(v/v) 2-mercapto ethanol, 2.3%(w/v) SDS, 62.5mM Tris/Cl pH6.8) for approximately one hour. The gel slices were then loaded directly into the sample wells of an SDS polyacrylamide gel and electrophoresis performed.

Protein Blotting

Proteins separated by PAGE were tansferred to nitrocellulose membranes using a Bio-Rad Transblot cell (Bio-Rad laboratories, Richmond, Cal., USA.). Gels were equilibrated in TrB for 30 to 45 minutes, depending on gel thickness, and transferred overnight at 30V (constant), 0.1A at 4°C in TrB.

Detection of Protein Kinase Activity on Nitrocellulose Membranes

After gel blotting, nitrocellulose membranes were incubated for 30 minutes in 0.5%(w/v) BSA in TBS. The saturated membranes were then washed three times in TBS. For labelling, the membranes were incubated in sealed plastic bags containing $+Ca^{2+}$ or $-Ca^{2+}$ buffer and approximately 370KBq [γ-^{32}P]ATP under the conditions indicated.

Upon completion, the membranes were washed overnight in TCA mix. After boiling for 15 minutes in fresh TCA mix and 30 minutes cooling, the membranes were washed a further two times with TCA mix and air dried.

ATP Analogue Affinity Labelling

^{14}C-5'-FSBA was stored as the stock solution, 0.5mM in 95%(v/v)

ethanol, at -20°C. When necessary this stock was evaporated to dryness under a stream of nitrogen at 0°C and resuspended in 1/10th initial volume of ethanol. Unlabelled 5'-FSBA was stored at 5mM in ethanol at -20°C.

For a time course of incorporation preparations were incubated at ~20°C in +/-Ca^{2+} and with ~45µM ^{14}C-5'-FSBA. At appropriate times aliquots were removed to cellulose discs and treated exactly as described for non-site specific protein kinase assays. Incorporated activity was then solubilised according to the method of Buhrow et al. (1982); the dry filters were treated with 0.5ml 10mM NaOH for 10 minutes and then 10ml of scintillation fluid 33%(v/v) Triton X100 in toluene with 4.7%(w/v) BPBD. Prior to counting the mixture was neutralized with 20µl of 1%(v/v) ethanoic acid.

Labelling of preparations for SDS-PAGE was performed as described for [γ-^{32}P]ATP, but with a 2 hour incubation at ~20°C using 0.45mM ^{14}C-5'-FSBA obtained by concentrating the stock solution. Incorporated activity was determined by scintillation counting of dry gel slices in 5ml of 4%(w/v) BPBD in toluene.

Electroelution of Protein From Non-denaturing Gels

Protein kinase containing bands were cut from the polyacrylamide gels and homogenised in a small volume of electrode buffer (25mM Tris/SO_4^{2-}, 20%(v/v) glycerol, pH8.0, ~200µl per 0.04cm³ of gel). The elution apparatus consisted of a 1ml (blue) disposible tip with the point removed. A small square of nylon mesh was placed across the cut tip and held in place by insertion into an eppendorf tube which had had the bottom and cap removed. Dialysis tubing filled with electrode buffer (see above) was slipped over the eppendorf tube and the free end knotted, bent back and held in place by a small rubber ring. The homogenised gel slices were loaded into the 1ml tip.

The unit was supported vertically between two tanks of electrode buffer (see above) with the dialysis tubing partially immersed in the lower tank. Elution was performed overnight with a constant 150V at 4°C.

At the end of the elution period the polarity was reversed for 5 minutes to reduce loss of protein caused by its tendency to stick to the dialysis tubing (Tijssen + Kurstak 1979). The small volume of eluate (~300μl) was recovered by slipping the dialysis tubing off the eppendorf tube.

Acid Hydrolysis of Proteins

The method used is based on that described by Tuy et al. (1983). Proteins were labelled as described for the 'basic assay' in non-site specific protein kinase assays. Reactions were terminated by addition of an equal volume of 30% (w/v) TCA, an equal volume of acetone and precipitation of protein on ice for 10 minutes. After centrifugation at 11,600 g for 10 minutes in an MSE Micro-Centaur centrifuge the supernatent was discarded and the pellet resuspended in 100μl of 0.1M NaOH. Following the addition of 50μl of 30%(w/v) TCA the protein was re-precipitated and the above washing repeated. The final pellet was washed with 100μl of acetone and resuspended in 100μl of 6M HCl. Hydrolysis was performed for 3 hours at 100°C in a Tecam (Techne, Cambridge, England.) DB-3 driblock. HCl was removed overnight by the hygroscopic uptake of NaOH pellets with gentle warming. The hydrolyzate was resuspended in ~10μl of a mixture of phosphoamino acids standards and carriers; $1mgml^{-1}$ of phosphoserine, phosphothreonine, phosphotyrosine, Na_2HPO_4, ATP and EDTA).

Two Dimensional Separation of Phosphoamino Acids

Hydrolyzed samples were spotted onto 20X20cm 100μm cellulose thin layer plates. The spot was concentrated and the plate evenly wetted

by overlaying with a Whatman 3MM template soaked in pH1.9 electrophoresis buffer (ethanoic acid:90%(v/v) methanoic acid:dH_2O - 78:24:898(v/v)) as described by Cooper et al. (1983). Electrophoresis in the first dimension was performed in pH1.9 buffer, with Whatman 3MM wicks, at 500V for 3.5 hours. After thorough drying, ascending chromatography in the second dimension was performed using 2-methyl-propanoic acid:'Ammonia' 0.5M - 5:3(v/v) for ~7.5 hours. Phosphoamino acid standards were visualised by ninhydrin staining.

Two Dimensional Separation of Phosphopeptides

Protein was labelled as described for protein kinase assays and reactions terminated by the addition of TCA (18.5%(w/v) final) and placing on ice for 15 minutes. Precipitated protein was pelleted by centrifugation at 11,600 g in an MSE Micro-Centaur centrifuge for 10 minutes. Supernatants were removed and washed, twice, in 100µl of diethyl ether to remove residual TCA. Protein was re-pelleted after each wash with a two minute centrifugation as above. The final pellets were resuspended in 10µl of 50mM NH_4HCO_3 buffer, pH7.8, and 2µl of 10mgml $^{-1}$ chymotrypsin free trypsin added. Digestion to equilibrium was performed overnight at ~20°C. Bromophenol blue, 1µl of 0.2%(w/v) was added as a marker and the digests spotted at a side centered position on a 20X20cm 100µm cellulose thin layer plate. Separation in the first dimension was performed in 1%(w/v) NH_4HCO_3 at 400V for two hours, the plates having been evenly wetted as described for phosphoamino acid separation (above). After air drying, separation in the second dimension was perfromed by ascending chromatography in butanol:pyridine:ethanoic acid:dH_2O - 15:10:3:12(v/v). Plates were then air dried and subjected to autoradiography.

Gel Hydrolysis

SDS polyacrylamide gels containing ^{32}P labelled proteins were hydrolyzed using the method developed by Cheng and Chen (1981). Hydrolysis consisted of a 30 minute incubation, with gentle shaking, in 2M NaOH at 50°C in a water bath (Grant Instruments, Barrington, Cambridge, England.). Prior to drying down the gels were equilibrated in Storage (see Coomassie blue staining of gels).

High Voltage Paper Electrophoresis

High voltage paper electrophoresis was performed at pH3.5 (pyridine:ethanoic acid:dH_2O - 1:10:189 v/v) on Whatman 3MM paper. Applied samples were concentrated with half strength pH3.5 buffer and electrophoresis performed for 1 hour at 3KV.

Sephadex G-100 Column Chromatography

Sephadex G-100 (standard grade) was swollen in PBS and packed into the column (length 23cm, diameter ~3mm) at a flow rate of ~3ml per hour PBS. All subsequent runs were performed at a flow rate of 2.5ml per hour PBS + 0.02%(w/v) NaN_3 and ~83µl fractions collected by time. Blue dextran and ATP were used to determine the void and total volumes respectively. The column was calibrated at intermediate points with horse heart cytochrome c (12.5KDa), soybean trypsin inhibitor (21KDa), ovalbumin (43KDa) and BSA (68KDa). Samples, in 7.5%(v/v) glycerol were introduced by layering onto the wet bed surface.

Fast Protein Liquid Chromatography (FPLC)

FPLC separation was performed using a Pharmacia (Uppsala, Sweden) system with a Superose 12 (10mmX30cm) column. Protein samples were added to the column in ~150µl of 50mM sodium phosphate buffer (pH7.2) and the separation performed with the same buffer at a flow rate of $0.5mlmin.^{-1}$. Fractions of 300µl were collected by time and absorbance

at 280nm monitored continuously.

Antibody Production

Antibodies from mice were kindly produced by Dr.J.D.Ansell, Department of Zoology, University of Edinburgh. Eight mice were used and each obtained the following doses of protein at approximately six week intervals:-

1) 100μg of acetone solubilised 'total' membrane protein.
2) 100μg of acetone solubilised 'total' membrane protein.
3) 15μg of acetone solubilised 'plasma membrane enriched' protein.
4) 35μg of acetone solubilised 'plasma membrane enriched' protein.

Test serum was obtained prior to injection four and final serum approximately four weeks after injection four.

Double Diffusion Assays

Ouchterlony double diffusion assays were carried out on 4X4cm plates with 4.5ml of McIlvaine's agar per plate; 1.2%(w/v) agarose, 0.9mM citric acid, 0.8mM Na_2HPO_4, 0.02%(w/v) NaN_3, pH7.0. Wells (4mm diameter) were cut, samples applied and pricipitin lines developed overnight at room temperature in a water saturated chamber. After a brief rinse in dH_2O the plates were overlaid with a prewetted sheet of Whatman 3MM paper, inverted on paper towels and a ~75g weight applied for ~15 minutes. After repeating the above the plates were soaked overnight in 2 to 3 changes of 0.1M NaCl with periodical agitation. Plates were again pressed as above and dried in a stream of warm air. Coomassie staining was performed as desribed for acrylamide gels.

Enzyme Linked Immunoelectro Transfer Blotting (EITB)

EITB was performed as described by Tsang et al. (1983) and in the Bio Rad Immuno Blot Assay kit. All reagents were of electro-immuno assay purity. After blotting, the nitrocellulose membranes were washed for 10 minutes in ATBS and then site saturated for 30 minutes

with 3%(w/v) gelatin in ATBS. Suitably diluted 1st antibody in ATBS with 1%(w/v) gelatin was applied for the stated time period. After a brief rinse in dH_2O the nitrocellulose membranes were washed for 20 minutes in two changes of ATBS and then second antibody (Goat-anti-Mouse peroxidase linked), diluted 1 in 3000 in ATBS with 1%(w/v) gelatin, was applied for one hour. The nitrocellulose membranes were then washed as for 1st antibody. Peroxidase activity was 'developed' using $3mgml^{-1}$ Bio Rad horse radish peroxidase development reagent in methanol. Immediately prior to use 20ml of this solution were added to 100ml of ATBS with 60µl of 30%(v/v) H_2O_2. Reactions were terminated by a 10 minute wash in two changes of dH_2O and the results photographed wet.

Protein Determination

The method of Bearden (1978) was employed with absorbance measurement at a single wavelength (595nm) and BSA as standard. Assays consisted of 0.5ml Bearden reagent plus 0.5ml of a suitably diluted preparation. Absorbance measurements were made on a Pye Unicam SP8-100 ultraviolet spectrophotometer.

Coomassie Blue Staining of Polyacrylamide Gels and Nitrocellulose

Polyacrylamide gels, regardless of thickness or the presence of SDS, were generally stained for ~15 minutes in staining solution; 0.2%(w/v) Coomassie Brilliant Blue R in 50%(v/v) methanol and 7%(v/v) ethanoic acid. Destaining consisted of a brief rinse and then ~30 minutes wash in destaining solution; 25%(v/v) ethanol, 8%(v/v) ethanoic acid. Background staining was then removed by an overnight wash in storage solution; 5%(v/v) methanol, 7%(v/v) ethanoic acid.

Proteins blotted to nitrocellulose membranes were stained for ~1 minute in 0.1%(w/v) Coomassie Brilliant Blue R, 50%(v/v) methanol and 10%(v/v) ethanoic acid. Destaining was performed using 50%(v/v)

methanol and 10%(v/v) ethanoic acid until bands were clearly visible. Background staining was then removed by overnight washing in storage as above.

Silver Staining of Polyacrylamide Gels

The method used is that described by Goldman et al. (1980). Gels were fixed for a minimum of 20 minutes in 50%(v/v) methanol plus 12%(v/v) ethanoic acid and SDS removed by three 10 minute washes in 10%(v/v) ethanol, 5%(v/v) ethanoic acid. After a 5 minute wash in 3.4mM Potassium Dichromate in 0.0032N HNO_3 followed by four 30 second washes in dH_2O the gels were loaded for 30 minutes in 12mM $AgNO_3$ with 5 minutes of intense fluorescent light at the start. Staining bands were developed by two rapid rinses followed by agitation in another volume of developer; 0.28M Na_2CO_3 in 0.05%(v/v) commercial formalin. Development was terminated by washing for at least 5 minutes in 1%(v/v) ethanoic acid. Gels were then washed in dH_2O prior to drying down.

Ninhydrin Staining of Thin Layer Plates and Electrophoresis Papers

Thin layer plates were sprayed evenly with 0.29%(w/v) ninhydrin in 97%(v/v) butanol, 3%(v/v) ethanoic acid and colour developed at room temperature. High voltage paper separations were treated likewise with 0.2%(w/v) ninhydrin in acetone.

Autoradiography and Fluorography

X-ray film was exposed directly to the labelled material at room temperature either in pre-designed cases or sandwiched between glass plates. On some occa sions a fluorescent screen was used to enhance the image and in these instances film was exposed to labelled material at -80°C (Laskey 1980).

RESULTS

THE POSSIBILITY OF A TYROSINE SPECIFIC PROTEIN KINASE ACTIVITY IN PEAS

In the introduction the importance of tyrosine phosphorylation, even as a minor component of the total protein kinase activity, was revealed. A general theme which is seen to emerge is the involvement of membrane bound/associated tyrosine kinases in cell growth control. However, all tyrosine kinases discovered at this time had been found in animal systems. No report of even a search for their presence in plants had been published. It should be noted that whilst many of the tyrosine kinases discovered in animals were products of retroviruses, thus representing an abnormal cell state, some are present in 'normal' cells e.g. the receptors for insulin, epidermal growth factor and platelet derived growth factor.

Protein kinase activity regulated by calcium ions had been demonstrated in membranes isolated from pea (Hetherington + Trewavas 1982), representing the first report of membrane bound and calcium regulated protein kinase activity in plants. No analysis of the amino acids phosphorylated by this enzyme activity had been made, and it was assumed to yield phosphoserine and phosphothreonine. Due to the apparent importance of tyrosine phosphorylation in animal cells it was considered necessary to check for such an activity in the membranes of Hetherington and Trewavas (1982).

The results presented below use a crude membrane preparation from pea buds (Hetherington + Trewavas 1984a), here referred to as 'total' membrane. An analysis of the amino acids phosphorylated by the protein kinase activity in the membranes is presented, combined with an extended search for a tyrosine specific component.

Hydrolysis of Labelled Total Protein and Two Dimensional Phosphamino Acid Analysis

Labelled total protein from 'total' membrane fractions was subjected to acid hydrolysis and two dimensional separation on cellulose thin layer plates. Phosphoamino acid standards, which were incorporated in the separation, were visualized by ninhydrin staining and labelled phosphoamino acids revealed by autoradiography. Figure 1 presents an autoradiograph produced by one such experimemt. A major portion of the phosphorylation is on serine residues, with a minor amount on threonine. No spot for phosphotyrosine is visible.

By spotting known amounts of [γ-^{32}P]ATP onto a separate thin layer plate, followed by autoradiography as above, it was estimated that ~20 disintegrations per minute could be detected in a spot of this size. Even after 'over-exposure' of the autoradiograph shown in Figure 1 no radioactive spot was seen to co-migrate with the phosphotyrosine marker. Two dimensional phosphoamino acid analyses, as described above, have also been carried out using 'total' membrane fraction from dark grown pea root, dark grown Zucchini hypocotyl and crown gall B6 callus tissue. Likewise, ammonium sulphate precipitated soluble fractions for all of the above tissues, including pea bud, have been tested and in no case was a spot for phosphotyrosine observed. In all cases phosphoserine represented the major portion of the phosphoamino acid content.

A recent report in the literature (Elliott + Geytenbeek 1985) claims the identification of phosphotyrosine in acid hydrolyzates of protein from ^{32}P labelled tobacco and crown gall transformed cells. However, the authors had to resort to scintillation counting of regions of the electrophoretogram since a discrete spot for phosphotyrosine was absent, as observed above for the *in vitro*

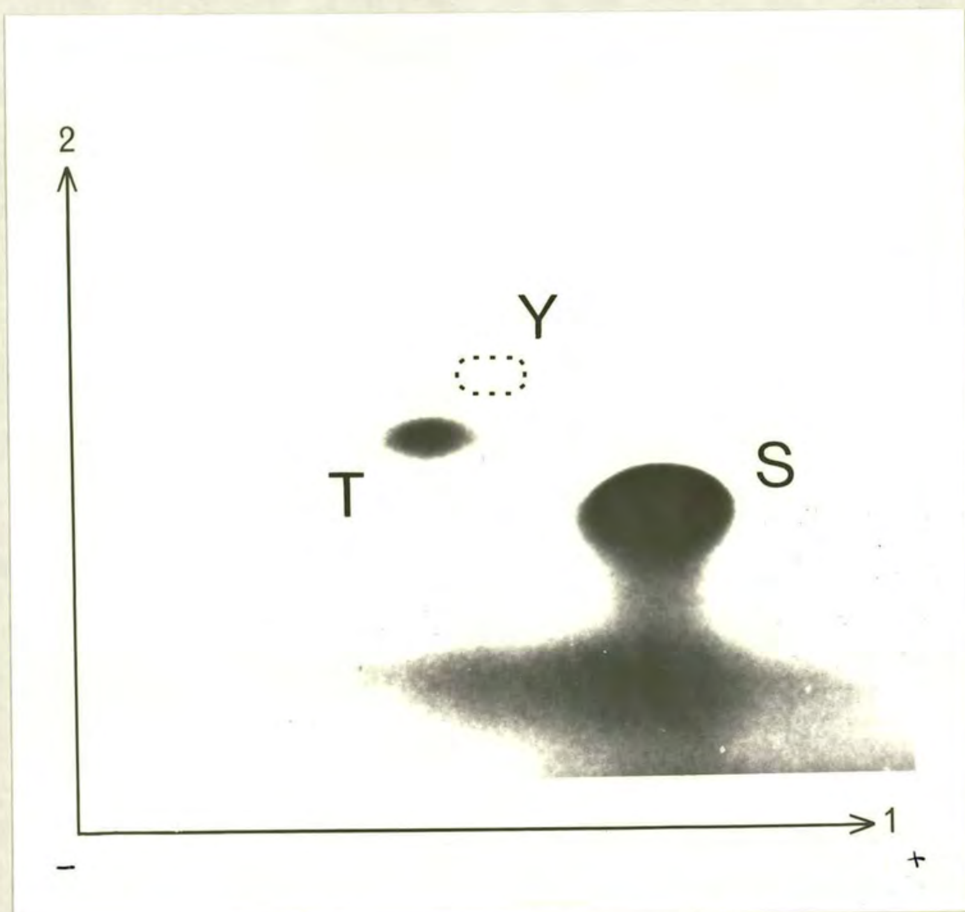

FIGURE 1.
Autoradiograph of a two dimensional separation of phosphoamino acids produced by protein kinase activity in a 'total' membrane preparation. A 'total' membrane fraction was isolated and labelled using [γ-^{32}P]ATP in the presence of ~100µM free calcium ions for 1 minute at ~4°C. Labelled protein was precipitated, hydrolysed in 6N HCl and subjected to two dimensional separation on a cellulose thin layer plate. 1st dimension - pH1.9 electrophoresis. 2nd dimension - ascending chromatography in 2-methyl-propanoic acid:ammonia. Positions of ninhydrin staining standards are indicated; S=phosphoserine, T=phosphothreonine and Y=phosphotyrosine. Only the relevant portion of the separation is shown.

analysis performed here. Such an analysis should be regarded with caution, especially in that very low counts were obtained and phosphothreonine will pass through the phosphotyrosine region in the system used by Elliott and Geytenbeek (1985). Whilst phosphotyrosine migrates ahead of phosphothreonine in the system presented in Figure 1 it was decided that alternative, and more sensitive, methods for the identification of phosphotyrosine should be tested.

Differential Stability of Phosphoamino Acids in Hot Alkali

Free phosphotyrosine has a relatively poor stability under acid hydrolysis conditions, as used above, when compared to phosphoserine and phosphothreonine. Loss is due to the hydrolysis of the phosphate ester linkage and is approximately 50% after five hours at 100°C in only 1M HCl (Cooper et al. 1983). Based on the data of Plimmer (1941) a method has been developed to subject ^{32}P labelled proteins separated by SDS-PAGE to hot alkali hydrolysis (Cheng + Chen 1981). Again considering the free phosphoamino acids, phosphotyrosine shows a remarkable stability in alkali with only 1% hydrolyzed after five hours at 100°C in 1M NaOH (Plimmer 1941). However, others have found that phosphothreonine, when present in certain proteins, can also show a degree of stability comparable to that of phosphotyrosine (Cooper + Hunter 1981). Alkali stable bands should, therefore, be subjected to phosphoamino acid analysis for certainty of identification.

'Total' membrane preparations were labelled using [γ-^{32}P]ATP in +Ca^{2+}, separated by SDS-PAGE and portions of the gels subjected to alkali hydrolysis. Labelled bands were then visualized in the dried down gel by autoradiography. Figure 2 shows the results of one such experiment. The unhydrolyzed lane is deliberately shown 'over-exposed' so as to indicate the absence of even minor bands in

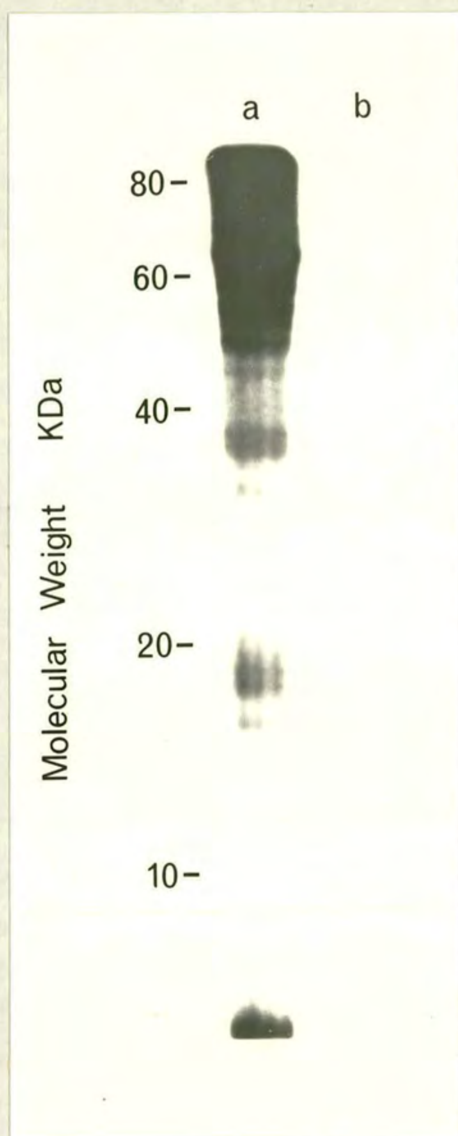

FIGURE 2.
Autoradiograph illustrating the effect of alkali hydrolysis on proteins labelled by protein kinase activity in a 'total' membrane fraction. A 'total' membrane fraction was isolated and labelled using [γ-^{32}P]ATP in the presence of ~100µM free calcium ions for 1 minute at ~4°C. After separation of labelled proteins by SDS-PAGE lane b was subjected to hydrolysis in 2M NaOH at 50°C for 30 minutes. After drying down the gel portions were autoradiographed together, lane a is deliberately shown over exposed to emphasise the lack of residual activity in lane b. Protein is not lost from the gel during hydrolysis.

the alkali treated portion. Whilst it might not be expected that all phosphorylation sites in a single band would be on tyrosine the level of phosphotyrosine in such crude preparations in clearly very low.

Arificial Substrates as a Means to Amplify Tyrosine Kinase Activity

Low levels of Phosphotyrosine could be the result of the presence of few sites suitable for phosphorylation. As an alternative to the detection of one of the products of such an enzymic reaction it is also possible to directly assay for tyrosine kinase activity itself. Infact the latter is a more unequivocal demonstration since phosphotyrosine not formed by kinase activity is found in biological systems (Mitchell + Lunan 1964 and Rothberg et al. 1978). The use of artificial substrates should supply the enzyme(s) with an excess of, but not necessarily ideal, sites for phosphorylation. Such substrates must be easily isolated/assayed and in this case must not contain serine or threonine. Both angiotensin II (an animal peptide hormone - Asp-Arg-Val-Tyr-Ile-His-Pro-Phe, Wong + Goldberg 1983) and tyramine (the decarboxylation product of tyrosine, Braun et al. 1983) are suitable and readily available substrates. Free tyrosine cannot be used as it has a very limited solubility in water. Even glycerol has been used with a degree of success, although at rather high concentrations (Braun et al. 1983). The phosphorylated products are isolated, after TCA precipitation of protein and hydrolysis of remaining [γ-^{32}P]ATP, by the method of Braun et al. (1983).

Such tyrosine specific protein kinase assays were performed using 'total' membrane in +Ca^{2+} in the presence of 2mM angiotensin II. Phosphate incorporated into TCA soluble material in the presence of 2mM angiotensin II was found to be only 61% of the control value with no angiotensin. Likewise the presence of 50mM tyramine reduced the

FIGURE 3.
Autoradiograph indicating the lack of angiotensin II phosphorylation by a 'total' membrane preparation. A 'total' membrane fraction was isolated and incubated with [γ-^{32}P]ATP and ~100µM free calcium ions in the absence (lane a) or presence (lane b) of 2mM angiotensin II for 1 minute at ~4°C. The TCA soluble products of the phosphorylation reactions were then separated by high voltage electrophoresis at pH3.5. After ninhydrin staining the paper was autoradiographed. The position of angiotensin II (Ang II) is shown in lane b.

TCA soluble incorporated phosphate to 52% of the control value. Increasing inhibition by tyramine was seen over the range 0mM to 50mM tyramine. Such inhibition is not unusual since Braun et al. (1984) found that several synthetic polymers with ordered sequences were potent inhibitors of isolated tyrosine specific protein kinases. Inhibition by tyramine has not been reported elsewhere.

In the above experiments the precise nature of the TCA soluble material phosphorylated is not known and the possibility existed that the artificial substrates were phosphorylated, whilst the phosphorylation of endogenous TCA soluble material had been inhibited. A check for the presence of phosphoangiotensin II and phosphotyramine was therefore made.

For the identification of phosphoangiotensin II the supernatants produced after TCA precipitation of protein in the tyrosine specific protein kinase assays were kept unhydrolysed. After extraction of TCA by mixing with 200µl of diethyl ether the aqueous phases were separated by high voltage paper electrophoresis at pH3.5. Figure 3 shows the results of one such separation for control and test samples.

Clearly there are no differences between the lanes, phosphoangiotensin II travels in the same direction as angiotensin II at this pH but migrating a shorter distance (Wong + Goldberg, 1983). Upon elution and acid hydrolysis of the labelled regions in each lane only free phosphate was obtained.

Results similar to the above were also obtained with an ammonium sulphate precipitated soluble fraction isolated from pea buds.

The identification of phosphotyramine required the production of a suitable marker. An attempt was made to decarboxylate purchased phosphotyrosine using tyrosine decarboxylase (EC 4.1.1.25) from Streptococcus faecalis. As this proved to be unsuccessful and Braun

et al. (1983) give no method for the identification of phosphotyramine the presence of unknown spots on two dimensional separations of the assay products was considered. No such spots were detected.

Whilst it is possible that peptides with sequences other than that of angiotensin II may be more suitable substrates, it seems that tyrosine kinase activity in the membrane system used here is below detectable levels.

Conclusion

The results presented above indicate that tyrosine specific protein kinase activity, and the presence of phosphotyrosine in proteins, is below the limits of detectability in the preparations tested. Phosphoserine is by far the major product of protein kinase activity. However, the presence of such activity in plants in general must remain an open question. In non-plant systems, techniques as described above have been used successfully with, where appropriate, both in vivo and in vitro labelling. No in vivo labelling has been performed in this investigation and an approach similar to that of Elliott and Geytenbeek (1985) may well have yielded positive results. The use of ATP for in vitro labelling also introduces a possible limiting factor, but an absolute specificity for another nucleotide triphosphate is unusual (Krebs + Beavo 1979). However, Shih et al. (1980) reported an absolute specificity for GTP in the autophosphorylation on threonine for a retroviral product.

Tyrosine kinase activity, if present, was clearly too low to allow further investigation. Attention was therefore focused on the isolation and characterisation of the protein kinase(s) responsible for the calcium dependent activity in the membranes of Hetherington and Trewavas (1984a).

LOCALIZATION OF CALCIUM ACTIVATED PROTEIN KINASE ACTIVITY IN THE PLASMA MEMBRANE

Calcium activated protein kinase activity had already been demonstrated in a crude membrane preparation from pea shoots (Hetherington + Trewavas 1982). By analysis of portions of the dark grown pea shoot (bud, apical hook, elongating zone and mature zone plus node) Hetherington and Trewavas (1984a) determined that protein kinase activity with the highest in vitro calcium activation could be obtained from the bud. In accordance with this analysis the present method of producing washed membranes from a 48,000 g pellet from pea buds was developed. Thus, the 'total' membrane preparation used in this work represents the optimized source of membrane associated, calcium activated, protein kinase from dark grown pea shoots. Little calcium dependent protein kinase activity was found in either nuclear rich or soluble fractions (Hetherington + Trewavas 1984a).

Using the density dependent methods of Rasi-Caldogno et al. (1982) to produce a fraction reported to be rich in plasma membrane, Hetherington and Trewavas (1984a) suggested that the calcium dependent protein kinase activity had a major location in this membrane type. The highest calcium activation and specific activity was detected in the plasma membrane enriched fraction when compared to 'heavier' membranes in their gradient. A major peak of protein and calcium activation of protein kinase activity was found at a density of 1.136gcm^{-3} upon isopycnic sucrose gradient centrifugation.

A more precise preparation of a plasma membrane fraction and more thorough characterization of its properties was required to unequivocally demonstrate that the calcium activated protein kinase activity had a major locale in this membrane type.

Phase Partitioning of 'Total' Membrane

Phase partitioning of membranes and organelles in aqueous two phase systems of polyethylene glycol and dextran was developed by Albertsson (1958). The separation is based on the surface characteristics of the particles (Larsson 1983), rather than density, and plasma membrane preferentially partitions into the upper polyethylene glycol rich phase (Larsson 1983). It is also reported (Larsson et al. 1984) that only 'right-side-out' sealed plasma membrane vesicles partition into the upper phase. Purity is therefore obtained at the expense of yield.

'Total' membrane was phase partitioned according to the methods of Yoshida et al. (1983) and each fraction assayed for calcium activated protein kinase activity. Figure 4 shows such results for the upper, 'plasma membrane enriched', phase and the lower, 'residual', phase (Blowers et al. 1985). 'Total' membrane gave results very similar to those for the 'residual' phase. Clearly, there are marked differences in the kinetics of labelling. On a specific incorporation basis, protein kinase activity is much higher (about six fold at four minutes) in the 'plasma membrane enriched' fraction and continues for a longer period of time. Such differences in activity are possibly due to the removal of phosphatases and proteins not directly associated with the kinase system partially purified in the 'plasma membrane enriched' fraction. It should be noted that only ~9% of the protein loaded partitions into the 'plasma membrane enriched' fraction (see Table 1). Calcium activation (~100μM free Ca^{2+}), estimated from separate results covering the first minute of Figure 4, was ~5.4 fold for the 'plasma membrane enriched' fraction. This activation compares favourably with that obtained (5.7 fold) by Hetherington and Trewavas (1984a) for a more crude plasma

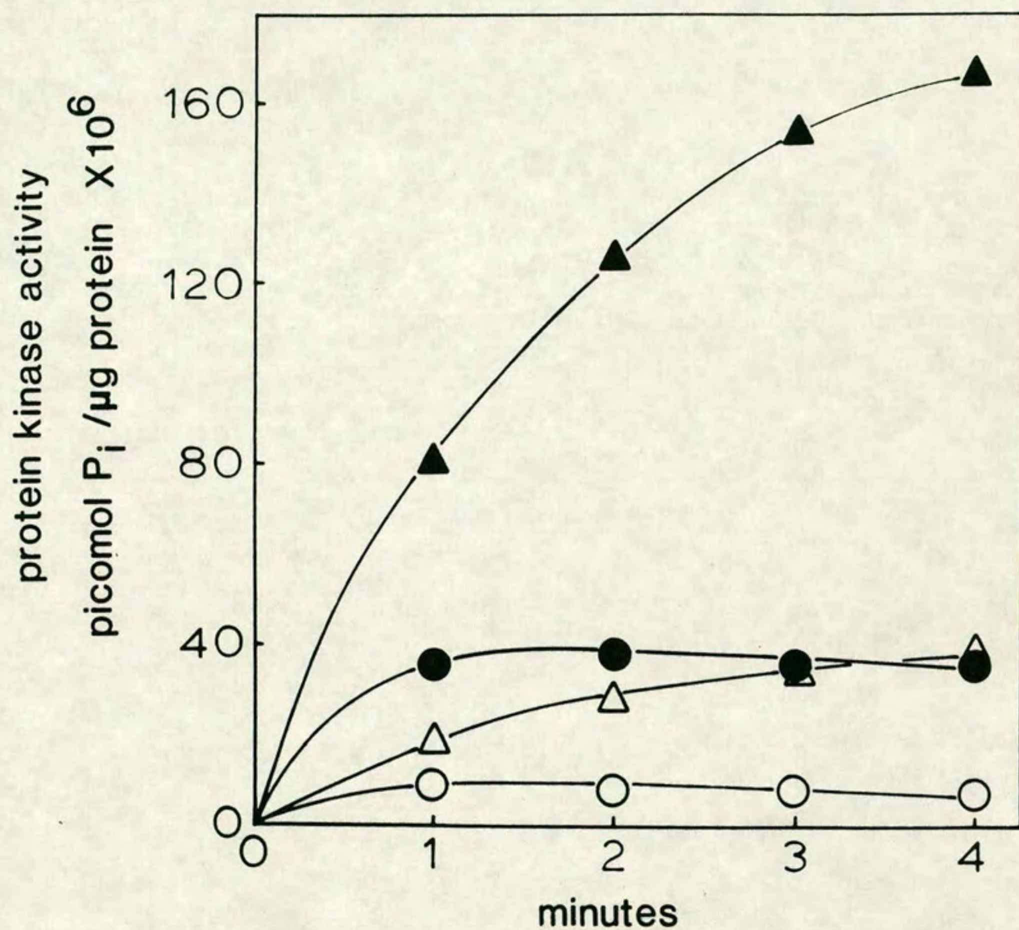

FIGURE 4.
Protein kinase activity in membrane fractions produced by phase partitioning of a 'total' membrane fraction. A 'total' membrane fraction was prepared and separated into 'plasma membrane enriched' (triangles) and 'residual' (circles) fractions. Protein kinase activity was assayed in each fraction in the presence (closed symbols) and absence (open symbols) of ~100µM free calcium ions with [γ-^{32}P]ATP at 0°C. Each point is the average of duplicate determinations.

membrane fraction.

SDS-PAGE of the Phase Partitioned 'Total' Membrane Fractions

To assess the pattern of proteins labelled in the 'residual' and 'plasma membrane enriched' fractions samples were incubated with [γ-^{32}P]ATP in the presence or absence of ~100µM free calcium and subjected to SDS-PAGE. Figure 5a shows an autoradiograph produced by these preparations. The activation by calcium in both preparations is readily apparent and several bands in the 'residual' fraction are seen to be represented to a lesser extent in the 'plasma membrane enriched' fraction. Conversely, other labelled bands are represented to a greater extent in the 'plasma membrane enriched' fraction, note particularly the band at ~18KDa.

Figure 5b shows the silver staining of the 'residual' and 'plasma membrane enriched' fractions after SDS-PAGE. The differences in protein content are clearly visible. Thus, a specific sub-set of proteins had been isolated by the phase partitioning technique. However, a demonstration of the identity of the upper phase as 'plasma membrane enriched' was necessary.

Isopycnic Sucrose Density Gradient Centrifugation of the Phase Partitioned 'Total' Membrane Fractions

To partially characterize the membrane fractions their distributions on a sucrose density gradient at equilibrium were determined (Yoshida et al. 1983). Figure 6 presents the profiles of protein obtained for, in this instance, the 'total' and 'plasma membrane enriched' fractions. Note the different scales used for protein content. The 'Plasma membrane enriched' fraction produces a single sharp peak with a corresponding density of 1.152gcm^{-3}. This is

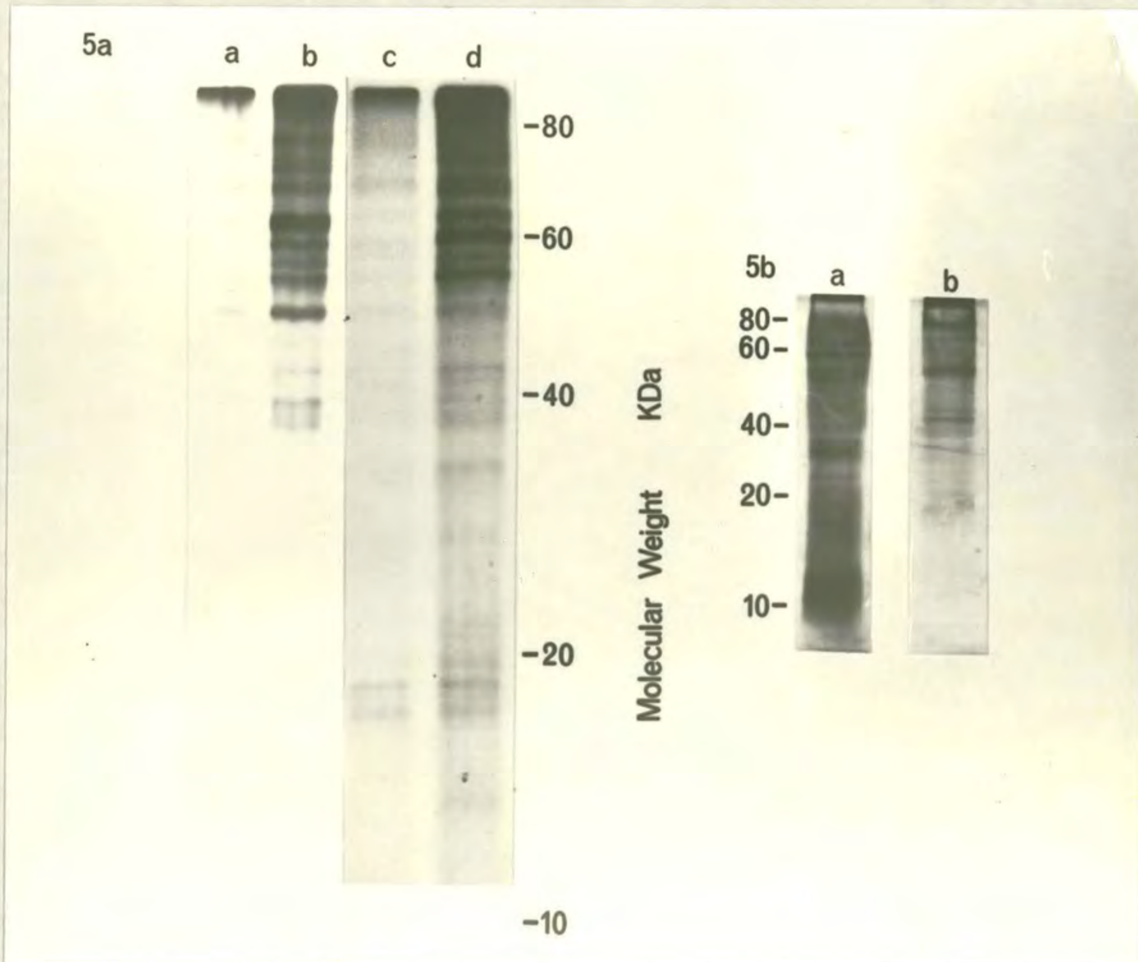

FIGURE 5a.
Autoradiographs showing the labelling of proteins due to protein kinase activity in fractions produced by phase partitioning of a 'total' membrane fraction. A 'total' membrane fraction was produced and phase partitioned into 'residual' (lanes a and b) and 'plasma membrane enriched' (lanes c and d) fractions. Proteins were labelled using [γ-^{32}P]ATP in the absence (lanes a and c) and presence (lanes b and d) of ~100μM free calcium ions for 1 minute at 0°C. Following separation of proteins by SDS-PAGE the gel was dried down and autoradiographed.

FIGURE 5b.
Silver staining of proteins in the membrane fractions produced by phase partitioning. 'Total' membrane was phase partitioned as above and the fractions separated by SDS-PAGE. The subsequent gel was silver stained. Lane a - 'Residual' membrane fraction. Lane b - 'Plasma membrane enriched' fraction.

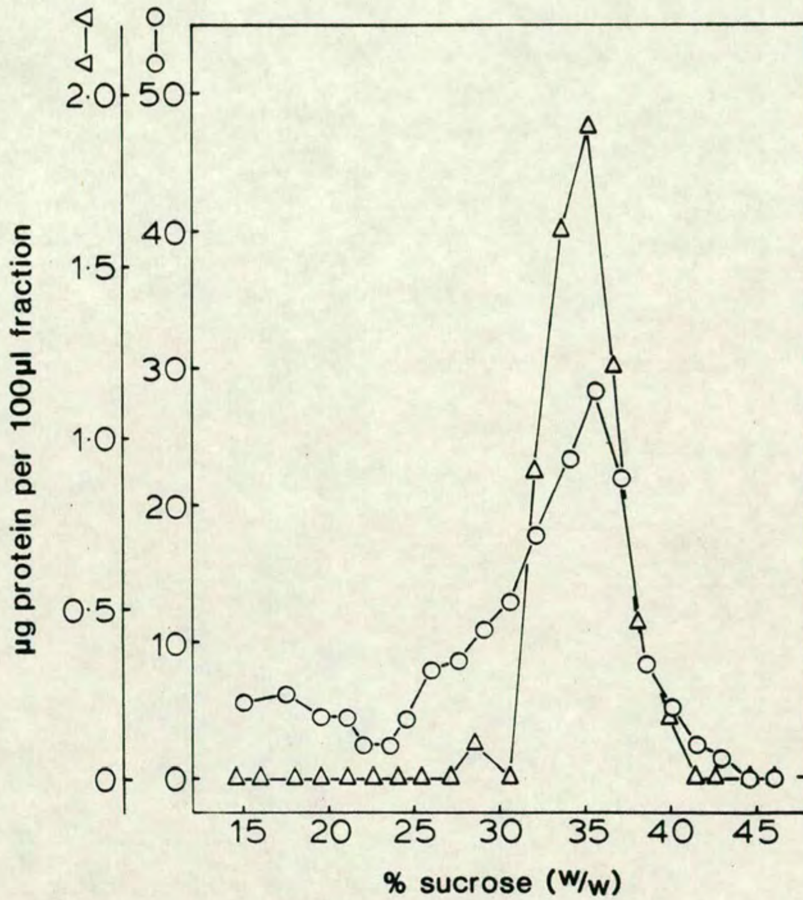

FIGURE 6.
Isopycnic sucrose density gradient protein profiles for a 'total' membrane (circles) and phase partitioning 'plasma membrane enriched' fraction (triangles). A 'total' membrane fraction was produced and phase partitioned to give a 'plasma membrane enriched' fraction. Membrane preparations were then loaded onto linear sucrose gradients and centrifuged to equilibrium densities. Upon fractionation of the gradients, protein assays were performed and sucrose content estimated with a refractometer.

higher than the unusually low values obtained by Hetherington and Trewavas (1984a) plus Hendrix and Pierce (1980), but is still within the typical range for plasma membranes (Sze 1985) and agrees favourably with that for Dactylis glomerata plasma membranes ($1.150 gcm^{-3}$) purified by the same method (Yoshida et al. 1983). 'Total' membrane is clearly very different, with a major peak at $1.158 gcm^{-3}$ and the presence of lighter membrane through to the top of the gradient.

In the buffer system used for the gradients the inclusion of 1mM EDTA should ensure that ribosomes are free from endoplasmic reticulum which would then be expected to show a mean density of $1.112 gcm^{-3}$(~26%w/w sucrose) (Lord 1983). Tonoplast will also band at such low densities (Sze 1985). It is therefore likely that the lighter membranes in the 'residual' fraction are endoplasmic reticulum and tonoplast.

Distribution of Marker Enzymes in the Phase Partitioned 'Total' Membrane Fractions

As a final characterization of the fractions produced by phase partitioning the distribution of some marker enzyme activities were tested. Three enzymes were chosen:- 1) NADH cytochrome c reductase, associated with both the endoplasmic reticulum and mitochondria as components of electron transport (Lord 1983), 2) Latent inosine diphosphatase (IDPase), associated with the golgi and thought to be due to inactivated polysaccharide synthetase activity (Ray et al. 1969) and 3) Cytochrome c oxidase, associated with electron transport in the inner mitochondrial membrane (Moore + Proudlove 1983).

The results for the marker enzyme assays are presented in Table 1 and the figures shown in brackets, after those for the 'plasma

	Protein	NADH Cytochrome c Reductase	Latent IDPase	Cytochrome c Oxidase
% distribution:				
Plasma membrane enriched	9 (~12)	3 (~3)	3 (~13)	1 (~8)
Residual	91	97	97	99
% Recovery	67	53	68	72

TABLE 1.
Distribution of marker enzyme activities and protein content for the 'plasma membrane enriched' and 'residual' phases produced by phase partitioning of a 'total' membrane fraction. The figures shown in brackets after the results for the 'plasma membrane enriched' fraction are equivalent data from Yoshida _et al._ (1983). Recovery is shown as the percentage of the activity in 'total' membrane that was obtained in the phase partitioned fractions. Figures are the average of duplicate determinations.

membrane enriched' fraction, are results taken from Yoshida et al. (1983). It is clear that the major portion of the activity for these marker enzymes partitions in the 'residual' fraction, as would be expected with the plasma membrane showing a much higher affinity for the other phase (Larsson 1983). Also shown in Table 1 is a recovery figure, this represents the fraction of the activity present in 'total' membrane that was obtained in the phase partitioned fractions. A likely source of loss is in the isolation of the very viscous lower phase (leading to its under representation in percentage terms), and the subsequent dilution and re-pelleting of each membrane fraction. It is, therefore, likely that the results presented in Table 1 under estimate the degree of purity obtained.

No unequivocal marker enzyme for the tonoplast has yet been described (Wagner 1983) and, therefore, no such data can be presented. Many supposed marker enzymes for the plasma membrane have been described, but most are regarded with caution (Hall 1983). The most promising to date would appear to be the vanadate sensitive ATPase described by Polonenko and Maclachlan (1984). However, since the system under purification in this case is an ATP hydrolysing one (protein kinase(s) and possible phosphatase(s)), such an activity would not make a suitable marker (Hall 1983). Finally, the separate partitioning of right-side and inside-out plasma membrane vesicles often leads to 'anomalous' data (Larsson 1983). Whilst cellulase was investigated as a possible marker no data for a plasma membrane marker is presented here.

Conclusion

Whilst other workers on plant calcium regulated protein kinases have studied membrane systems (Hetherington + Trewavas 1982; Polya et

al. 1984; Salimath + Marme 1983; Veluthambi + Poovaiah 1984) in only one case (Teulieres et al. 1985) has the activity been directly associated with a single membrane type, in this case the tonoplast. Polya et al. (1984) present initial data for a plasma membrane preparation. Thus, the demonstration of a plasma membrane location for a calcium regulated protein kinase is novel. Whether the calcium effect is regulated via calmodulin, or another calcium binding protein, is the subject of a later chapter. However, it should be noted that activation by calcium alone suggests the presence of an endogenous calcium binding protein. As assayed by NAD kinase activation it is estimated that 0.4% of the total protein in 'total' membrane is calmodulin (Hetherington + Trewavas 1984a). This calmodulin is tightly bound and does not dissociate in an EGTA wash. Whilst no calmodulin assays have been performed for the 'plasma membrane enriched' fraction it is assumed that a similar situation exists here.

Whilst a higher degree of purification would have been obtained with repeated partitioning using freshly synthesised lower phases, the preparation obtained here clearly demonstrates the location of a calcium regulated protein kinase activity. Such a preparation represents a useful starting point for further purification of the enzyme(s).

SOLUBILISATION OF PROTEIN KINASE ACTIVITY FROM MEMBRANE PREPARATIONS

The results presented in Chapter 1 show the purification of calcium dependent protein kinase activity whilst still membrane associated. In the course of isolating the protein kinase its solubilisation is inevitable. Such a procedure would hopefully eliminate a great number of proteins as possible candidates for the enzyme itself, but they may be substrates.

Attempted solubilisation of protein kinase activity from a crude membrane preparation from pea buds had already been reported by Hetherington and Trewavas (1984a). Their results using 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), nonidet P-40 (NP40) and octyl glucose indicate the ineffectiveness of these compounds. Initial evidence for acetone precipitation and solubilisation of the membrane proteins had also been obtained (Hetherington + Trewavas 1984a). Membrane proteins are precipitated with acetone and then resuspended in an aqueous buffer followed by centrifugation to remove insoluble protein (here referred to as acetone solubilisation). It was estimated that ~30% of the total protein kinase activity was solubilised and it still retained calcium activation (Hetherington + Trewavas 1984a). Such preparations could be inhibited by trifluoperazine (TFP) a calmodulin inhibitor (50% inhibition at ~50µM) suggesting co-solubilisation of calmodulin or a calmodulin like protein. A further analysis of this useful technique was required and its application to the 'plasma membrane enriched' fraction of Chapter 1 desirable since it would constitue a rapid means of further purification.

Acetone Solubilisation of Protein Kinase Activity from 'Total' Membrane

Using a modification of the method of Venis (1977) 'total' membrane protein kinase was solubilised with the acetone treatment and the product incubated with [γ-^{32}P]ATP in +/-Ca^{2+} prior to separation by SDS-PAGE. An autoradiograph produced by one such experiment is presented in Figure 7, lanes b and c (from Blowers et al. 1985) along with a coomassie blue stained lane. The retention of calcium activation is readily apparent, with only one band at ~60KDa showing labelling in the absence of free calcium. However, the degree to which activation by calcium was retained was on a few occasions not so apparent. Rather than minimal activity in the absence of calcium, the activity became virtually independent of calcium, suggesting that the enzyme(s) had been modified in some way or a variation in the calmodulin content of the preparation. Figure 7, lanes d and e, show an autoradiograph for the SDS-PAGE separation of labelled proteins in one such preparation. There are clearly marked differences when compared to lanes b and c, although the labelling of some bands is still dependent upon the addition of free calcium.

In terms of the total amount of protein in the initial membrane preparation only 1/20th was obtained in the final aqueous fraction. A critical point in this procedure was the rapid centrifugation after resuspension of the acetone pellet. Extension of the rehydration time from 15 to 45 minutes resulted in a ~50% decrease in protein recovery. The solubilised preparation was relatively unstable and after standing on ice for a few hours protein was seen to precipitate.

A comparison of Figure 7 with Figure 5a indicates that some labelled bands present in whole membrane ('residual' and 'total' membrane give virtually identical labelling patterns) are absent in

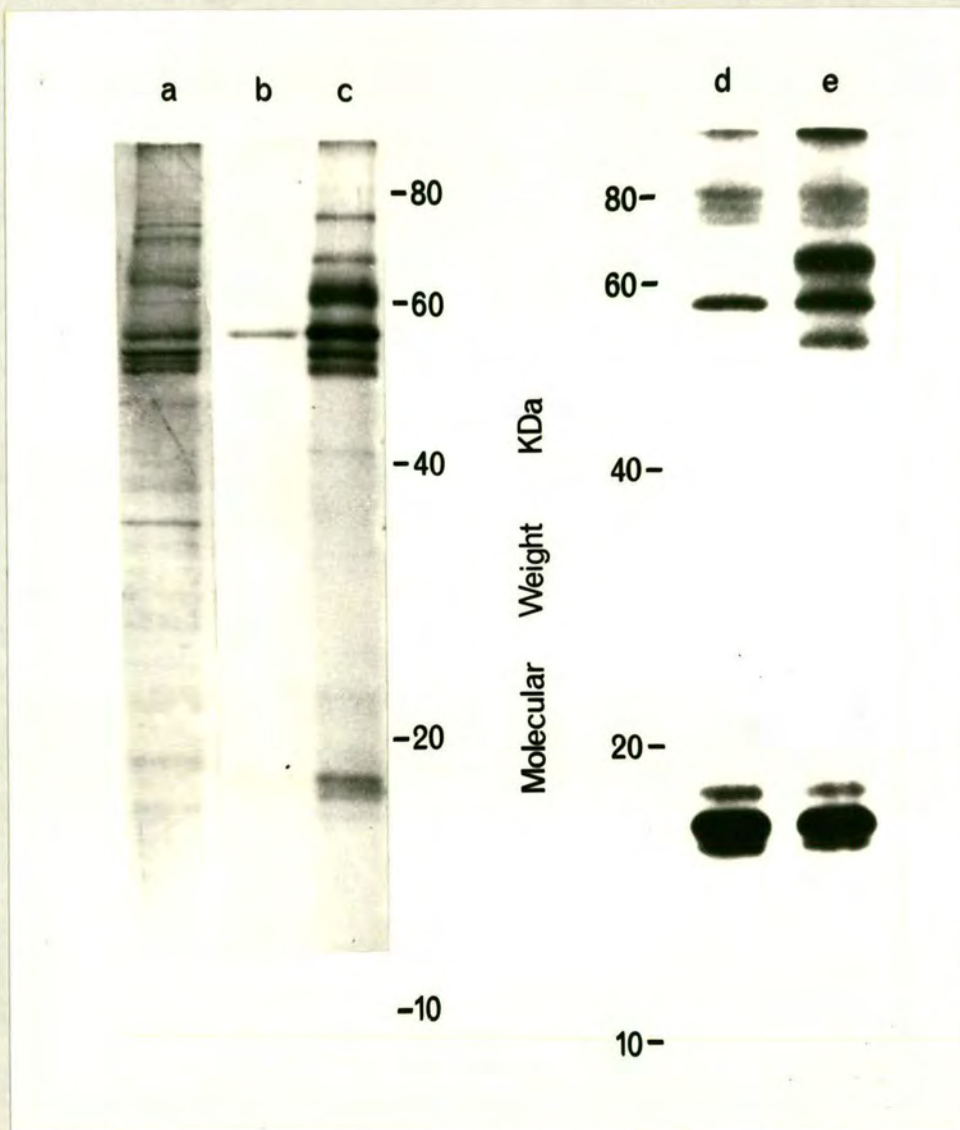

FIGURE 7.
SDS-PAGE analysis of acetone solubilised fractions of 'total' membrane preparations. A 'total' membrane fraction was produced and subjected to acetone precipitation and resuspension in an aqueous buffer (acetone solubilisation). To assess the proteins labelled by solubilised protein kinase activity the preparations were labelled using [γ-^{32}P]ATP in the absence (lanes b and d) and presence (lanes c and e) of ~100μM free calcium ions for 1 minute at 0°C. After separation of proteins by SDS-PAGE the dried down gels were autoradiographed. Lane a shows the coomassie blue stained gel for lane b. Lanes b+c and d+e are derived from separate preparations.

the solubilised form. It is likely that the latter represent the more hydrophobic substrates not soluble in the aqueous buffer. Note particularly the labelling around 18KDa in Figure 7, so far such labelling had only been observed in the 'plasma membrane enriched' fraction produced by phase partitioning (see Chapter 2).

Acetone Solubilisation of Protein Kinase Activity from a 'Plasma Membrane Enriched' Fraction

A membrane preparation enriched in plasma membrane was produced in Chapter 2 and evidence indicated that calcium activated protein kinase activity had a major location in this fraction. It was considered that the above acetone solubilisation of calcium activated protein kinase activity should be applied to achieve further purification of the enzyme(s).

Figure 8a presents an autoradiograph of acetone solubilised 'plasma membrane enriched' fractions labelled using [γ-^{32}P]ATP under three conditions. A single major band (~18KDa) is seen to be labelled in each lane, with calcium activation and virtually no effect of added calmodulin. A very minor labelled band at ~19KDa was observed on some occasions. As above, a decrease in the apparent calcium activation of the enzyme was observed in some preparations.

Figure 8b shows the acetone solubilised 'plasma membrane enriched' fraction separated by SDS-PAGE and silver stained. Many bands are visible and the protein kinase(s) is/are presumably amongst them. A comparison of this to Figure 5b, showing silver staining for 'residual' membrane (essentially identical to 'total') gives a clear indication of the small number of proteins remaining.

Unlike the preparation from 'total' membrane this solubilised fraction was remarkably stable. Several hours standing on ice and

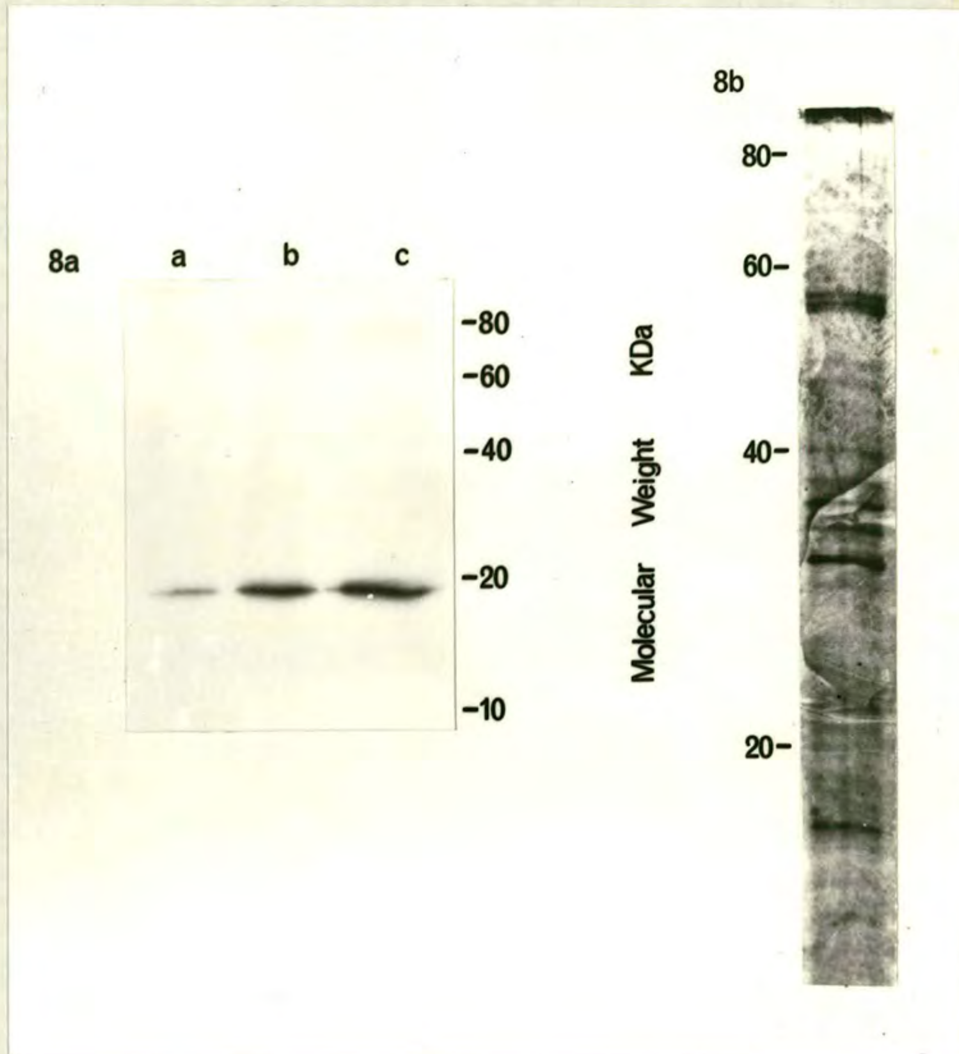

FIGURE 8a.
SDS-PAGE analysis of proteins labelled by protein kinase activity in an acetone solubilised 'plasma membrane enriched' fraction. A 'total' membrane preparation was produced and phase partitioned to give a 'plasma membrane enriched' fraction. This membrane fraction was then subjected to acetone solubilisation. Proteins were labelled by incubation with [γ-^{32}P]ATP in the absence (lane a) or presence (lanes b and c) of ~100μM free calcium ions for 1 minute at 0°C. In lane c bovine calmodulin is also present at 50μg per ml. Preparations were separated by SDS-PAGE and the dried down gel autoradiographed.

FIGURE 8b.
Protein composition of an acetone solubilised 'plasma membrane enriched' fraction. An acetone solubilised 'plasma membrane enriched' fraction was produced as above. After separation by SDS-PAGE the proteins were silver stained.

many quick freeze/thaw cycles did not affect its activity to any great extent.

Conclusion

Acetone treatment of membranes clearly provides a rapid means of removing calcium activated protein kinase activity from the membrane enviroment, although the yield is rather low. The apparent variation in the acetone solubilisation method was not necessarily a problem and the reason(s) for this variation remain to be established.

For the acetone solubilised 'plasma membrane enriched' fraction it is important to note that, essentially, only a single substrate is present and for both solubilisations that the enzyme(s), whilst soluble in aqueous buffer, is/are operating in a totally alien enviroment. However, that the ~18KDa band is also observed in the 'plasma membrane enriched' fraction suggests that its labelling is not totally non-representative of the 'intact' situation. No other workers on calcium regulated protein kinases in plants have published any material on solubilisation of membrane types. Comparisons cannot, therefore, be made.

The use of ~100μM free calcium was sufficient to fully activate the protein kinase in pea shoot membranes (Hetherington + Trewavas 1982), and 10μM was adequate. The use of ~100μM free calcium with both the phase partitioning fractions and the solubilised preparations here is assumed to be sufficient for full activation of the protein kinase.

The question now remains - which of the proteins solubilised above is/are calcium activated protein kinases?

IDENTIFICATION OF A PROTEIN KINASE IN ACETONE SOLUBILISED MEMBRANE PREPARATIONS

Thus far the purest preparation of the calcium activated protein kinase(s) available is the acetone solubilised 'plasma membrane enriched' fraction produced in Chapter 3. The problem now was to identify the protein kinase(s) amongst the many proteins present. The most obvious method would be the use of a suitable ATP analogue capable of binding covalently to the ATP binding site of the enzyme(s). Clearly, any other enzymes using ATP in the preparation would also be possible targets. Alternatively, the renaturation *in situ* of preparations separated by SDS-PAGE and subseqeunt detection of activity using exogenous substrates and [γ-^{32}P]ATP could be used, although the likelihood of retaining enzyme activity would be low in this case. Finally, though not so desirable, is the running of preparations in non-denaturing polyacrylamide gels and then identifying activity using [γ-^{32}P]ATP. In this case it is probable that protein complexes would be obtained intact, useful if necessary for enzymic activity, but not desirable if artifactual.

The following sections present results for all three of the above methods. The section on renaturation of proteins after SDS-PAGE has been separated into two parts, to allow for the inclusion of work on non-denaturing gels.

ATP Affinity Analogue Labelling

The ATP analogue used in this work was 5'-Fluorosulfonylbenzoyladenosine (5'-FSBA) and was available 'cold' or ^{14}C labelled at carbon 8 of the adenine moiety. A sulfonyl fluoride group takes the place of the terminal phosphate of ATP and acts as an electrophilic agent capable of reacting with several

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classes of amino acids (Colman et al. 1977). It should be noted that 5'-FSBA may also be considered as a reasonable analogue for ADP and NAD(H) (Colman et al. 1977).

All experiments using the ATP analogue with the acetone solubilised 'plasma membrane enriched' fraction were unsuccessful and, therefore, the work presented below relates to the acetone solubilised 'total' membrane preparation.

A time course of incorporation of ^{14}C-5'-FSBA was established for the acetone soulubilised 'total' membrane preparation. Aliquots removed from the incubation were assayed as for membrane protein kinase assays on cellulose discs since it was found that phosphocellulose gave very variable background counts with the ATP analogue. Discs were then counted in scintillation fluid, after solubilisation by the method of Buhrow et al. (1982), and the results of one such experiment are presented in Figure 9. It can be seen that incorporation is linear over a two hour period both in the presence and absence of calcium, although the counts are rather low. The slight effect of ~100μM free calcium is possibly a conformational effect on either protein or the analogue. Incubation times longer than two hours were not desirable due to the instability of the solubilised membrane preparation.

Two questions had now to be answered. To which protein(s) was the ^{14}C-5'-FSBA binding and did the binding of 5'-FSBA inhibit the calcium activated protein kinase activity? Firstly, acetone solubilised 'total' membrane was incubated with ^{14}C-5'-FSBA in the presence of ~100μM free calcium for 2 hours and then subjected to SDS-PAGE. Incorporated counts were so low that an autoradiograph could not be produced even with pre-flashed film and an enhancer in the gel. Figure 10 presents the results of scintillation counting of

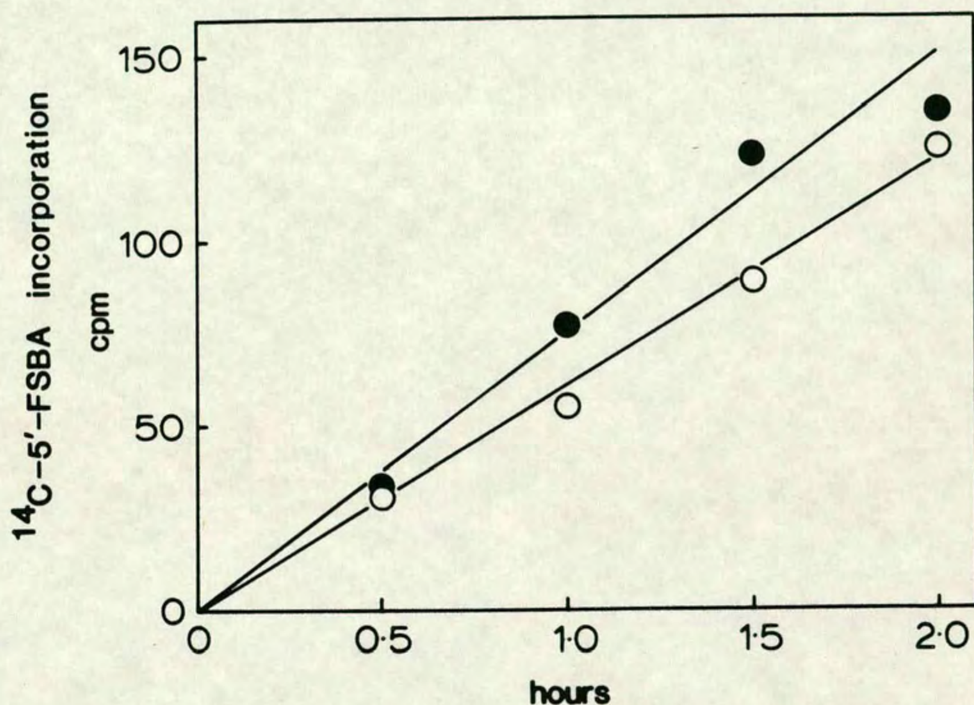


FIGURE 9.
Time course of ^{14}C-5'-FSBA binding to an acetone solubilised 'total' membrane preparation. A 'total' membrane preparation was produced and subjected to acetone solubilisation. The preparation was then incubated with ~45μM ^{14}C-5'-FSBA in the absence (open symbols) or presence (closed symbols) of ~100μM free calcium ions at 20°C. At the appropriate times aliquots were removed to cellulose discs and incorporated radioactivity estimated by liquid scintillation counting. Each point is the average of duplicate determinations.

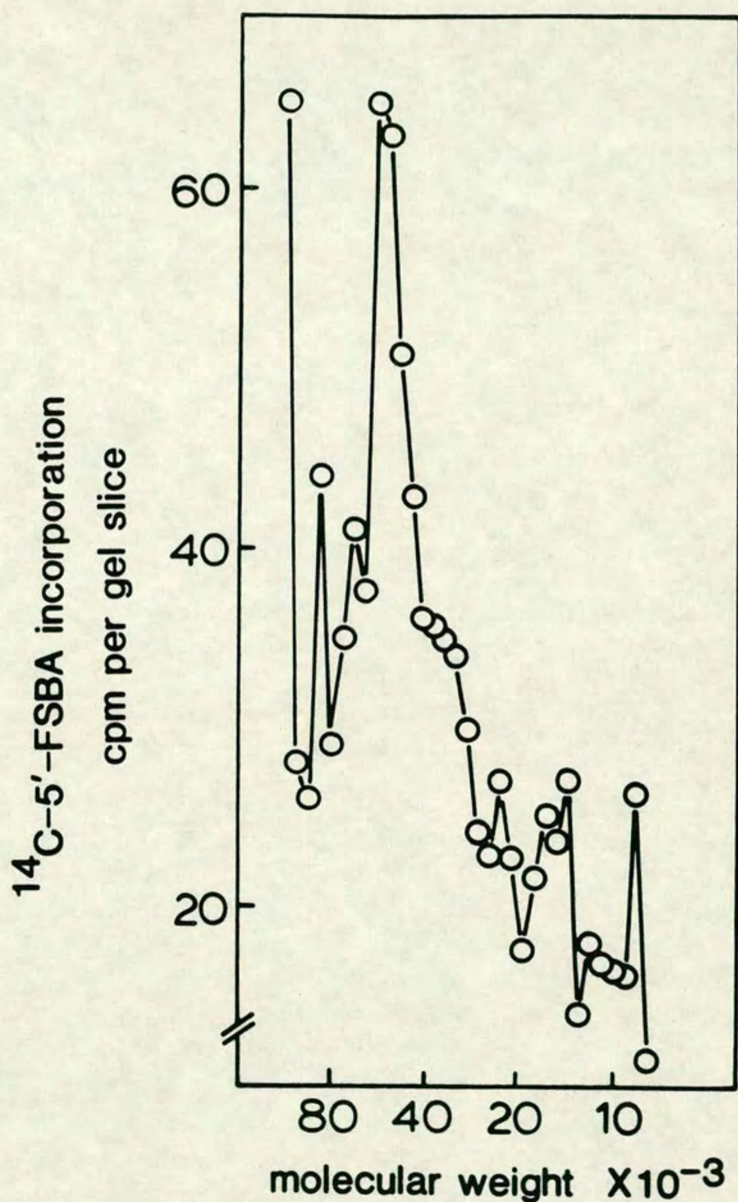


FIGURE 10.
SDS-PAGE distribution of ^{14}C-5'-FSBA labelled proteins in an acetone solubilised 'total' membrane preparation. A 'total' membrane preparation was produced and subjected to acetone solubilisation. The preparation was then incubated with 0.45mM ^{14}C-5'-FSBA in the presence of ~100μM free calcium ions for 2 hours at 20°C. After separation by SDS-PAGE incorporated activity was estimated by liquid scintillation counting of dried down gel slices.

gel slices from such a gel. Against a generally decreasing background a major peak is seen at ~55KDa, corresponding to a major staining protein in this preparation (see Figure 7). Thus, a protein apparently with an ATP binding site, possibly a protein kinase, is present in the acetone solubilised 'total' membrane preparation. It should be noted that a protein is also present at this molecular weight in the acetone solubilised 'plasma membrane enriched' fraction (see Figure 8b) and binding was probably not detected due to the small amount of protein present in this preparation.

Next the necessary attempt to inhibit protein kinase activity with 'cold' 5'-FSBA was made. Acetone solubilised 'total' membrane was incubated for various lengths of time in the presence of ~100μM free calcium and 5'-FSBA. By comparison with a suitable control without 5'-FSBA it was possible to determine any reduction in protein kinase activity with time as a result of 5'-FSBA. Such inhibition was very slight, with the highest estimate being 11% inhibition after 1 hour with 0.45mM 5'-FSBA. In comparison Buhrow et al. (1982) achieved ~60% inhibition of EGF stimulated protein kinase activity after a 1 hour treatment of A431 cell membranes with only 40μM 5'-FSBA.

Whilst it was possible that the protein kinase had been detected (~55KDa) the affinity of the labelled band for the analogue was very low. Further experiments were required to confirm or negate the above result.

Fixing and Renaturation of Proteins in SDS Polyacrylamide Gels

This section presents the first of two attempts to detect protein kinase activity after renaturation in SDS polyacrylamide gels. The method employed is a modification of that used by Kuret and Schulman (1985) and depends upon the property of numerous protein kinases to

autophosphorylate (see introduction). SDS is removed, the proteins renatured and fixed in the gel by extensive washing in a propan-2-ol/ethanoic acid/water mixture. A similar procedure is used in calmodulin overlay-type experiments (Carlin et al. 1981). After washing the gel with dH_2O, and $+Ca^{2+}$ it was transferred to $+Ca^{2+}$ with bovine calmodulin and [γ-^{32}P]ATP. Following the 4 hour incubation unincorporated radioactivity was removed by washing in TCA and pyrophosphate. Experiments of this type were carried out using both 'total' and 'plasma membrane enriched' acetone solubilised fractions. Autoradiographs of such gels failed to reveal any discrete bands, only a general non-specific background.

At this time no vastly different published methods were available, so non-denaturing gel electrophoresis was the only alternative.

Detection of Protein Kinase Activity after Non-Denaturing PAGE

Initially, the pattern produced by pre-labelled preparations on non denaturing gels was investigated. It will be seen below that such lanes act as suitable 'markers'. Both 'total' and 'plasma membrane enriched' acetone solubilised fractions were labelled using [γ-^{32}P]ATP in $+Ca^{2+}$. After separation by non-denaturing PAGE an autoradiograph was produced from the dried down gel. Figure 11, lanes b and e, show the results for the 'plasma membrane enriched' and 'total' phases respectively. Discrete bands of activity are present and the preparations appear to have only one band in common.

The demonstration of protein kinase activity in non-denaturing gels would involve lengthy washing steps and, since they contained only 7% acrylamide, diffusion would be a major problem. An attempt was therefore made to retain possible enzyme activity after blotting

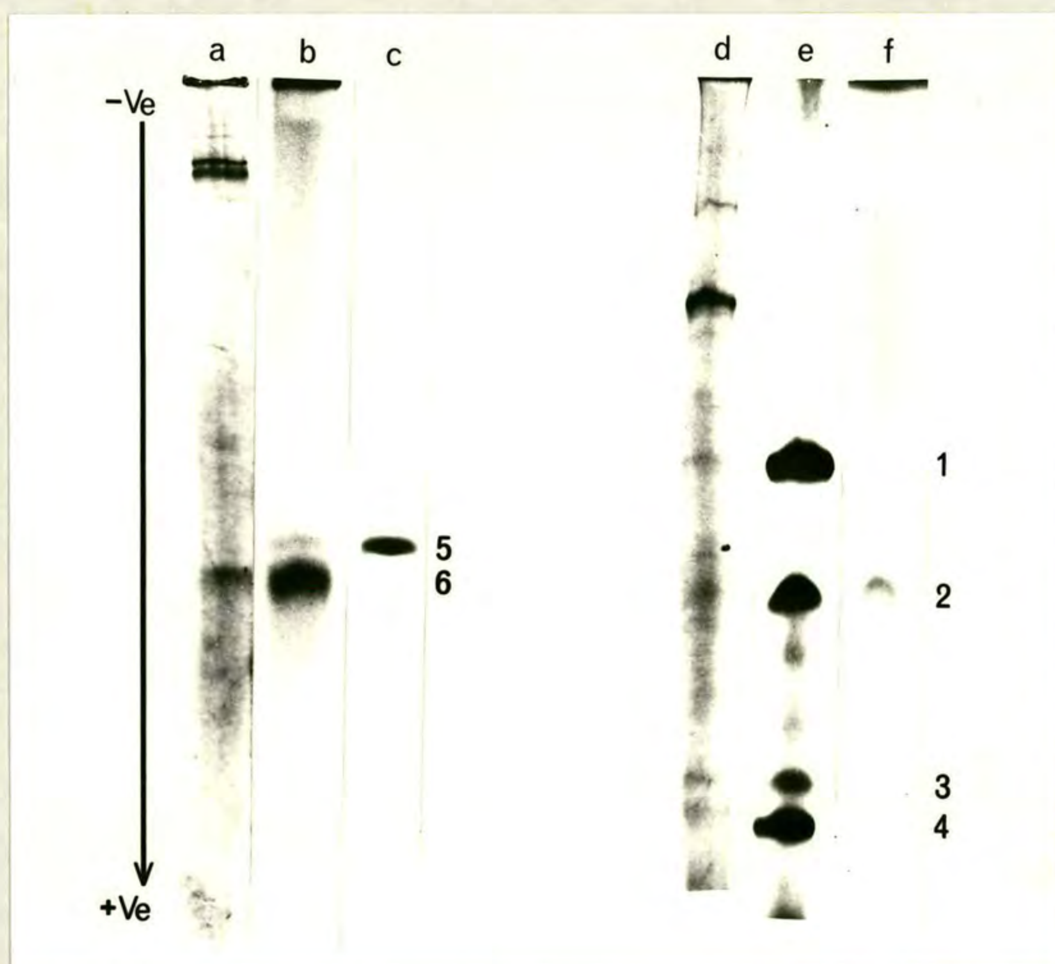


FIGURE 11.
An analysis of protein kinase activity in acetone solubilised 'plasma membrane enriched' (lanes a, b and c) and 'total' membrane (lanes d, e and f) fractions by non-denaturing PAGE and Western blotting. A 'total' membrane preparation was produced and phase partitioned to give a 'plasma membrane enriched' fraction. Both membrane fractions were then subjected to acetone solubilisation and proteins labelled using [γ-^{32}P]ATP prior to separation by non-denaturing PAGE or after blotting to nitrocellulose.

Lane a - Silver stained gel for lane b.

Lane d - Coomassie blue stained gel for lane e.

Lanes b+e - Preparations were labelled using [γ-^{32}P]ATP in the presence of ~100μM free calcium ions for 1 minute at 0°C and then separated by non-denaturing PAGE. The gel was dried down and autoradiographed.

Lanes c+f - Preparations were separated by non-denaturing PAGE and blotted to nitrocellulose. Protein kinase activity was then detected by incubation of the blots with [γ-^{32}P]ATP for 2 hours at 20°C in the presence of ~100μM free calcium ions. The hot TCA washed nitrocellulose was then dried and autoradiographed.

The numbers by the labelled bands relate to Figure 13.

of such gels to nitrocellulose (initial success in this respect was obtained by Dr.A.Hetherington). After site saturation of the nitrocellulose the incubation with [γ-^{32}P]ATP in +Ca^{2+} and extensive washing was performed. A single labelled band, in the same location, was detected for both of the solubilised membrane fractions (Figure 11, lanes c and f). Such a band is also observed in the prelabelled preparations (Figure 11, lanes b and e). Blotting of a prelabelled lane along with an unlabelled lane enabled the precise alignment of the 'enzyme' band in the latter.

To ensure that the labelled band produced by the nitrocellulose blotted preparations was due to protein kinase activity, rather than non-specific association of [γ-^{32}P]ATP or ^{32}P (both unlikely to survive the hot acid washing), a phosphoamino acid analysis was performed (thanks are due to Dr.A.Hetherington and Dr.J.Somerville for performing this analysis). Labelled bands, as in Figure 11 lane f, were excised and subjected to acid hydrolysis. Figure 12 (from Blowers et al. 1985) shows a pH1.9 high voltage electrophoretic separation of the products. Phosphoamino acids are clearly present with a major portion of the activity directed towards serine, agreeing with the results presented in Chapter 1. The identity of the minor spot has not been further resolved, although the results presented in Chapter 1 would suggest this to be phosphothreonine.

Thus, the position of a protein kinase on non-denaturing gels had been determined. However, whilst very probable, it was not known with certainty whether this enzyme was responsible for the calcium acivated activity observed in the 'total' and 'Plasma membrane enriched' fractions. Chapter 5 investigates this point further.

By alignment of autoradiographs from portions of non-denaturing gels it was possible to excise the labelled bands from wet gels. Such

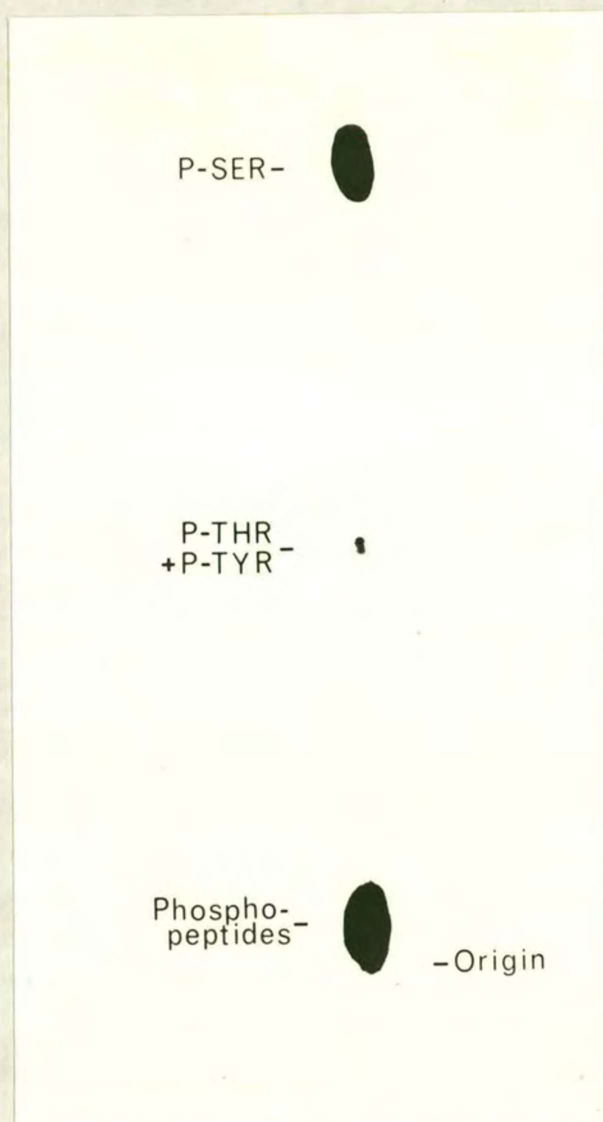


FIGURE 12.
Autoradiograph of amino acids labelled by nitrocellulose-bound protein kinase activity. A 'total' membrane preparation was produced and subjected to acetone solubilisation. After separation by non-denaturing PAGE and blotting to nitrocellulose the protein kinase containing band was labelled in the presence of ~100µM free calcium ions using [γ-^{32}P]ATP. The subsequent labelled band was excised and hydrolysed in 6N HCl and phosphoamino acids separated by high voltage paper electrophoresis at pH1.9. The paper was ninhydrin stained, to reveal standards, and autoradiographed. P-SER, phosphoserine; P-THR, phosphothreonine; P-TYR, phosphotyrosine. Thanks are due to Dr.A.Hetherington and Dr.J.Somerville for this figure.

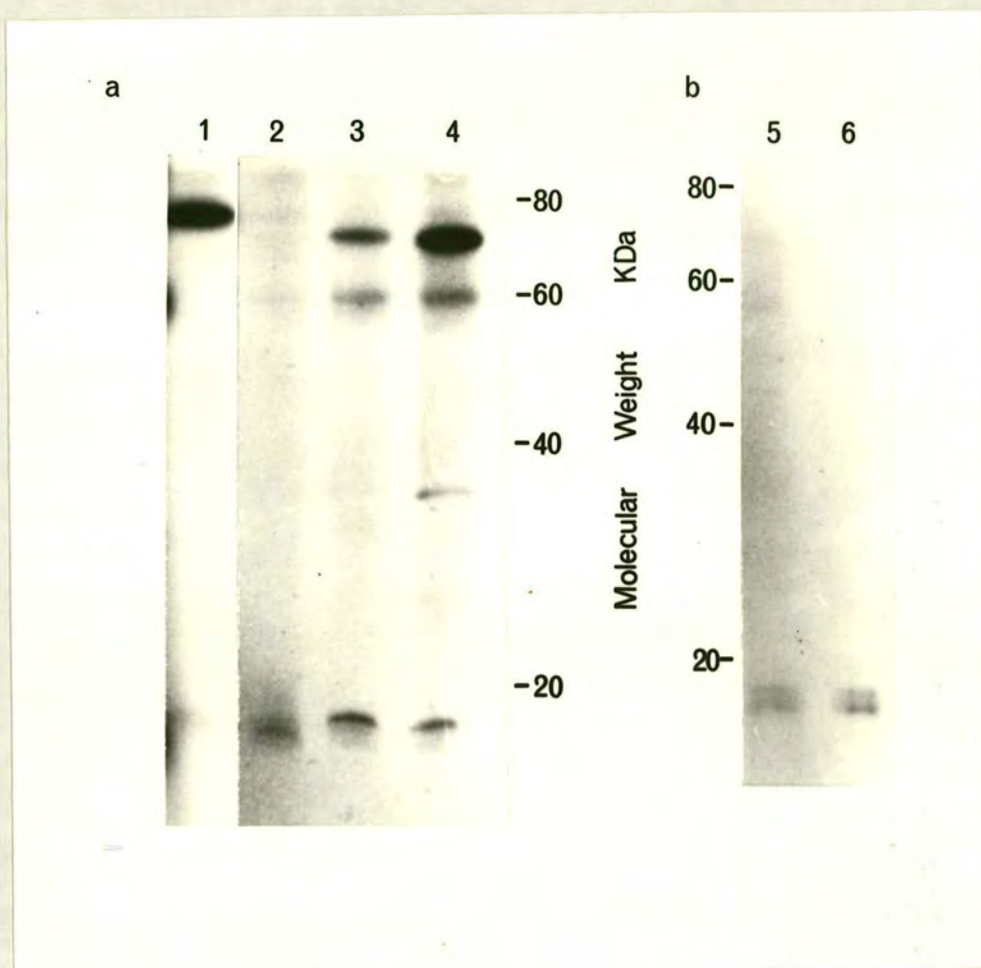


FIGURE 13.
An examination of the labelled protein content of labelled bands derived from non-denaturing PAGE separations of the acetone solubilised 'total' membrane (a) and acetone solubilised 'plasma membrane enriched' (b) fractions. A 'total' membrane preparation was produced and phase partitioned to give a 'plasma membrane enriched' fraction. Both membrane fractions were then subjected to acetone solubilisation. Proteins were labelled using [γ-^{32}P]ATP in the presence of ~100μM free calcium ions and separated by non-denaturing PAGE. Labelled bands were then excised and subjected to SDS-PAGE. The dried down gels were autoradiographed. The numbered lanes correspond to the bands of Figure 11.

bands were then subjected to SDS-PAGE to analyse their labelled peptide content. Autoradiographs produced by this type of analysis are presented in Figure 13. Figure 13a shows the results for the bands derived from the acetone solubilised 'total' membrane preparation. For the band in non-denaturing PAGE which was seen to phosphorylate when blotted to nitrocellulose (labelled as number 2) only a single band at ~18KDa can be seen in the SDS-PAGE separation. All of the other non-denaturing bands are seen to be composites of labelled bands observed in an SDS-PAGE separation of the acetone solubilised 'total' membrane preparation.

Figure 13b shows an equivalent analysis for the the two bands produced by the acetone solubilised 'plasma membrane enriched' fraction upon non-denaturing PAGE. In each case only one or two band at ~18KDa can be seen. A discussion of these observations is given in the conclusion for this chapter.

A Modified Method for Renaturation of Proteins in SDS Polyacylamide Gels

Having completed all of the above work a novel method for detecting protein kinases in SDS polyacrylamide gels appeared in the literature (Geahlen et al. 1986). The method involves short renaturation times, avoids fixing proteins in the gel and includes an exogenous substrate in the gel matrix. The latter may not only act as a substrate, but aids renaturation (Geahlen et al. 1986). Histone H1 had been found to be a suitable substrate for the enzyme (see Chapter 7, Figure 27) and Figure 14 shows an autoradiograph produced by a renatured SDS polyacrylamide gel containing acetone solubilised 'total' and 'plasma membrane enriched' fractions after $[\gamma-^{32}P]$ATP treatment and washing to remove non-covalently bound ^{32}P and

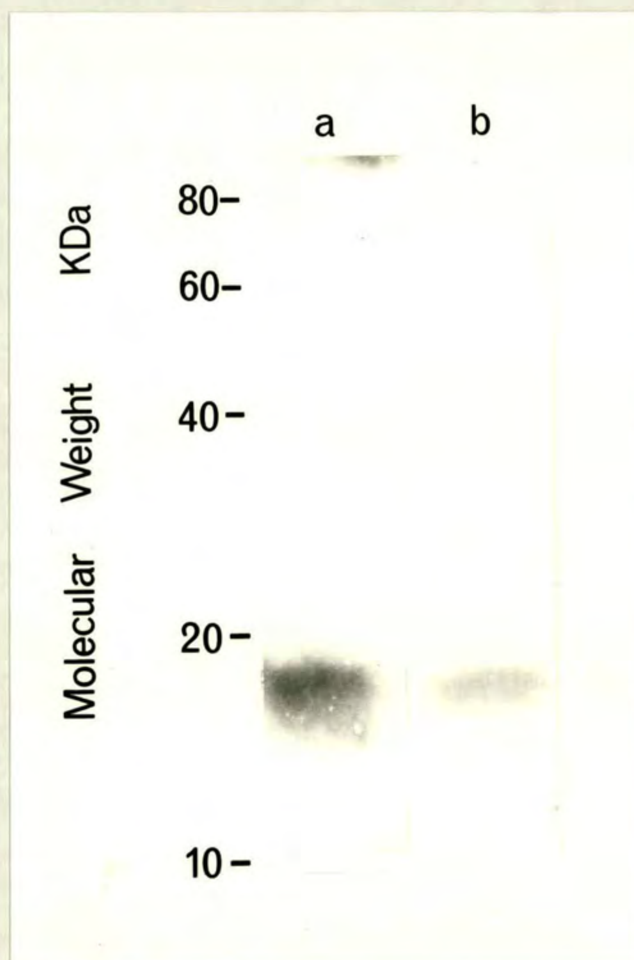


FIGURE 14.
Protein kinase activity in solubilised membrane preparations renatured after SDS-PAGE. A 'total' membrane preparation was produced and phase partitioned to give a 'plasma membrane enriched' fraction. Both membrane fractions were subjected to acetone solubilisation and separation by SDS-PAGE in a gel containing histone H1. After removal of SDS by washing in a HEPES buffer protein kinase activtiy was detected by incubation with [γ-^{32}P]ATP in the presence of ~100µM free calcium ions and 100µg per ml bovine calmodulin for 1 hour at 4°C. The extensively washed and TCA treated gel was dried down and autoradiographed. Lane a - acetone solubilised 'plasma membrane enriched' fraction. Lane b - acetone solubilised 'total' membrane fraction.

[γ-^{32}P]ATP. A single, slightly diffuse, band of ~18KDa can be seen in each lane. This result confirmed that the ~18KDa band had protein kinase activity and that it could autophosphorylate, since it was essentially the only labelled band present when labelled preparations of acetone solubilised 'plasma membrane enriched' fraction were subjected to SDS-PAGE. A portion of the phosphorylation in the renatured gel is likely to be autophosphorylation, the remainder being phosphorylated histone. Whether such autophosphorylation is inter- or intra-molecular is examined in Chapter 7.

Conclusion

The data provided by the use of an ATP analogue is unsatisfactory, but its binding to one protein (~55KDa), and possibly a trace of binding at ~18KDa, should not be ignored. It is possible that another protein kinase or ATPase of some kind has been detected. No investigation of the binding of 5'-FSBA to membrane bound proteins has been performed since the objective was to identify a protein kinase in a solubilised preparation. It remains to be seen whether the ~18KDa protein would bind 5'-FSBA in an experiment using 'plasma membrane enriched' fraction.

Importantly, a labelled band of ~18KDa is observed in 'plasma membrane enriched' fraction (Figure 5a), acetone solubilised 'total' membrane (Figure 7) and acetone solubilised 'plasma membrane enriched' fraction (Figure 8a). However, this band is not as apparent in 'total' membrane and 'residual' membrane, its lack of detection in this preparation is probably due to its low percentage content, enhanced in acetone solubilisation. The presence of an ~18KDa band does not guarantee a common identity for all, peptide mapping or the use of antibodies (see Chapter 8) would validate such observations.

However, autophosphorylaton has been demonstrated in this region for both the 'total' and 'plasma membrane enriched' acetone solubilised fractions (Figure 14) suggesting a common identity.

With reference to the detection of protein kinase activity after separation by non-denaturing PAGE it is interesting to note that some bands containing the enzyme did not autophosphorylate when blotted to nitrocellulose, this is clearly illustrated by the results presented in Figure 13. Closer inspection of the protein kinase band produced by autophosphorylation of the acetone solubilised 'total' membrane preparation on nitrocellulose reveals it to be located towards the top of the band produced by a prelabelled preparation (see Figure 11, lanes e and f). A much clearer demonstration of this phenomenon is illustrated by lanes b and c of Figure 11, for the acetone solubilised 'plasma membrane enriched' fraction. Of the two bands present in lane b only the upper appears upon labelling of the blotted preparation (lane c). SDS-PAGE analysis revealed the ~18KDa band to be the only labelled protein in both of these bands (Figure 13b). Why should only one of these bands appear upon labelling of proteins blotted to nitrocellulose? With the knowledge that the protein kinase autophosphorylates it is possible that phospho/dephospho forms of the enzyme have been separated. The enzyme as isolated, presumably in a relatively unphosphorylated state, runs as the upper band of Figure 11 lane b. However, <u>in vitro</u> autophosphorylation of the enzyme possibly shifts its running position in non-denaturing PAGE to that of the lower band in Figure 11 lane b. In this instance only the upper band is capable of demonstrating autophosphorylaton when blotted to nitrocellulose, as illustrated by Figure 11 lane c. The faint labelling of the upper band in Figure 11 lane b is a possible indication of the lower level of phosphorylation. It is not known

whether autophosphorylation shifts the apparent molecular weight of the enzyme upon SDS-PAGE analysis, as found by Yamauchi and Fujisawa (1985).

A similar argument to that presented above can also be applied to the acetone solubilised 'total' membrane preparation on non-denaturing PAGE, although in some cases the phosphorylated enzyme would appear to be associated with other substrates.

Variation in the degree of phosphorylation of the isolated enzyme could possibly explain the results presented and discussed in Chapter 3, where differences in the properties of the solubilised enzyme are considered.

Whilst not isolated to homogeneity, protein kinase activity has been associated with a specific protein on a polyacrylamide gel. Its apparent ability to autophosphorylate and its activation in this respect by calcium and calmodulin is the subject of the following chapters.

ELECTROELUTION OF THE PROTEIN KINASE AND DEMONSTRATION OF CALMODULIN ACTIVATION

In the previous chapter a protein kinase present in the acetone solubilised 'total' and 'plasma membrane enriched' fractions was isolated to a single band on a non-denaturing gel. In both cases the only substrate present in these bands was the enzyme itself i.e. autophosphorylation was observed. An analysis and discussion of autophosphorylation is presented in Chapter 7. To assess any possible calcium and calmodulin activation of the autophosphorylation the enzyme containing band was electroeluted from non-denaturing gels. By using a pre-labelled lane as a marker it was possible to bulk up slices from unlabelled gel portions and electroelution was performed at pH8.0 since this gave a known mobility direction of the protein(s) as determined in non-denaturing PAGE. By counting radioactivity in the initial gel and final eluate it was estimated that this process recovered only ~50% of the protein and since a large portion of the total amount of enzyme is likely to be lost in the phase partitioning step (see Chapter 2) the acetone solubilised 'total' membrane fraction was preferred to acetone solubilised 'plasma membrane enriched' fraction. The results presented below concern the electroeluted band from acetone solubilised 'total' membrane fraction.

Protein Content of the Electroeluted Protein Kinase

The electroeluted protein kinase containing band from the acetone solubilised 'total' membrane fraction was subjected to SDS-PAGE and silver staining to examine its protein content. A photograph of one such silver stained gel is presented in Figure 15 (lane a). Clearly more than one protein, although not substrate (see below), is present and it is worthwhile making a comparison to a coomassie blue stained

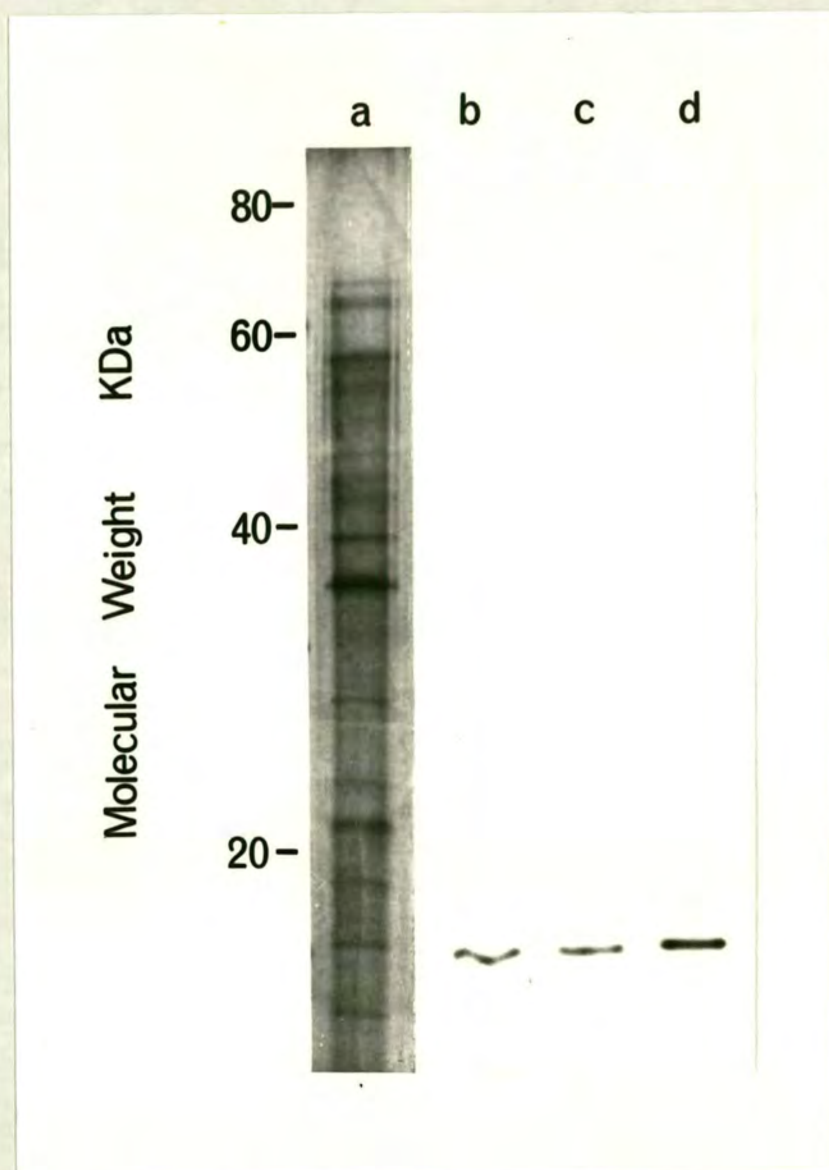


FIGURE 15.
SDS-PAGE analysis of the electroeluted protein kinase fraction and demonstration of calcium plus calmodulin activated autophosphorylation. A 'total' membrane preparation was produced and subjected to acetone solubilisation. After separation by non-denaturing PAGE the autophosphorylating protein kinase containing band was located by comparison with a separate lane labelled using [γ-^{32}P]ATP. The protein kinase containing band was electroeluted from homogenised gel slices. Lane a shows the electroeluted protein kinase fraction separated by SDS-PAGE and silver stained. Lane b, c and d show preparations labelled using [γ-^{32}P]ATP in the absence of free calcium (lane b), in the presence of ~100μM free calcium ions (lane c) and as lane c but with 50μg per ml bovine calmodulin (lane d). Preparations were labelled for 1 minute at 0°C. After separation of the labelled preparations by SDS-PAGE the dried down gel was autoradiographed.

lane for the acetone solubilised 'total' membrane fraction as shown in Figure 7. Whilst the relative intensities and specificities of staining for each type of procedure must be borne in mind it is readily apparent that a subset of the total starting protein has been isolated in the electroeluted fraction.

Calmodulin Activation of the Electroeluted Protein Kinase

In every preparation of the enzyme so far the presence of 100μM free calcium alone has led to a marked increase in protein kinase activity, presumed to be mediated by the presence of endogenous calmodulin or another calcium binding protein (see conclusion of Chapter 2). Figure 16 (from Blowers et al. 1985) shows the results of time courses for protein kinase activity in the electroeluted protein kinase from acetone solubilised 'total' membrane. Very similar results were also obtained with the electroeluted band from the acetone solubilised 'plasma membrane enriched' fraction. In contrast to all results obtained up to this point there is virtually no activation in the presence of ~100μM free calcium alone. However, the addition of ~100μM free calcium with bovine calmodulin at 50μg per ml (~3μM) leads to an approximate three fold increase in initial rate and what would appear to be a higher plateau for specific incorporation. In longer term experiments this difference is maintained indicating a higher equilibrium phosphorylation level in the presence of calcium and calmodulin. The addition of calmodulin alone had little effect and this result is not likely to be a non specific protein-protein interaction. Figure 15 (lanes b, c and d) shows an autoradiograph for labelled proteins in each of these assays when separated by SDS-PAGE. In each case only a single major band at ~18KDa is present, with a very minor band at ~19KDa, and the calmodulin activation of

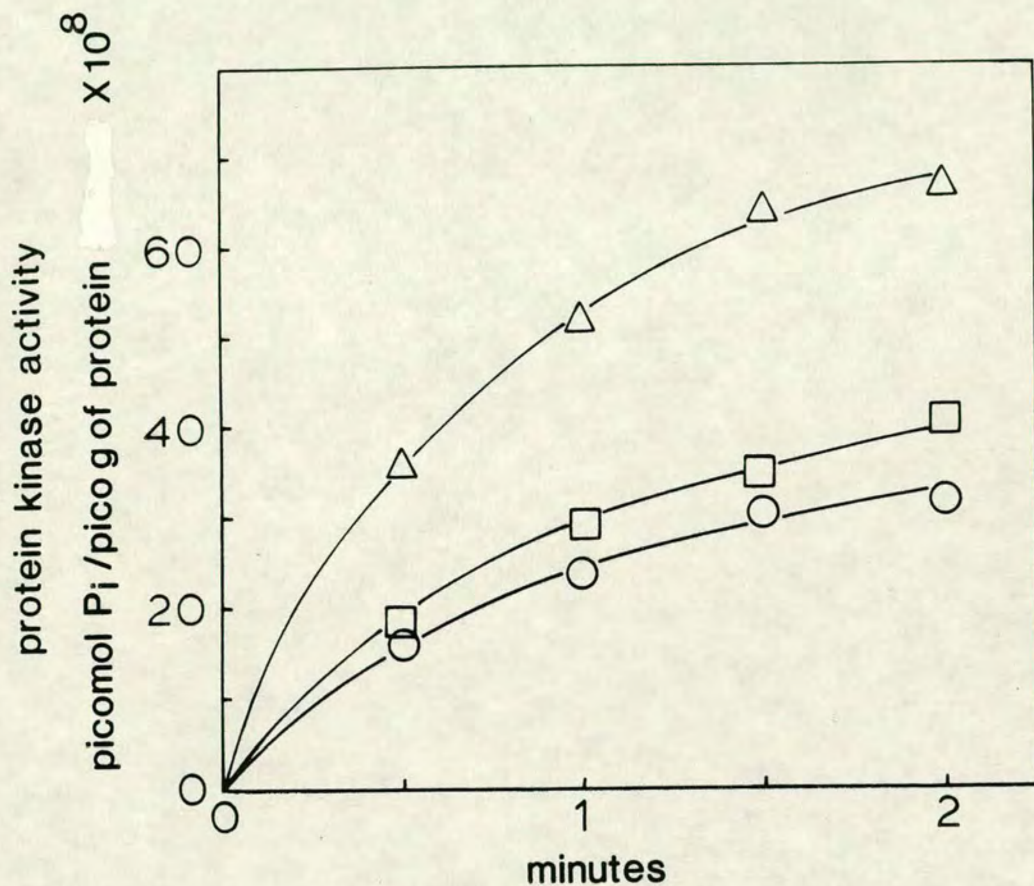


FIGURE 16.
Calcium and calmodulin activation of protein kinase activity in the electroeluted fraction. A 'total' membrane preparation was produced and subjected to acetone solubilisation. After separation by non-denaturing PAGE the autophosphorylating protein kinase containing band was located by comparison with a separate lane labelled using [γ-^{32}P]ATP. The protein kinase containing band was electroeluted from homogenised gel slices. The subsequent eluate was assayed for protein kinase activity using [γ-^{32}P]ATP in the absence of free calcium (circles), the presence of ~100μM free calcium ions (squares) and ~100μM free calcium ions with 50μg per ml bovine calmodulin (triangles). Each point is the average of duplicate determinations.

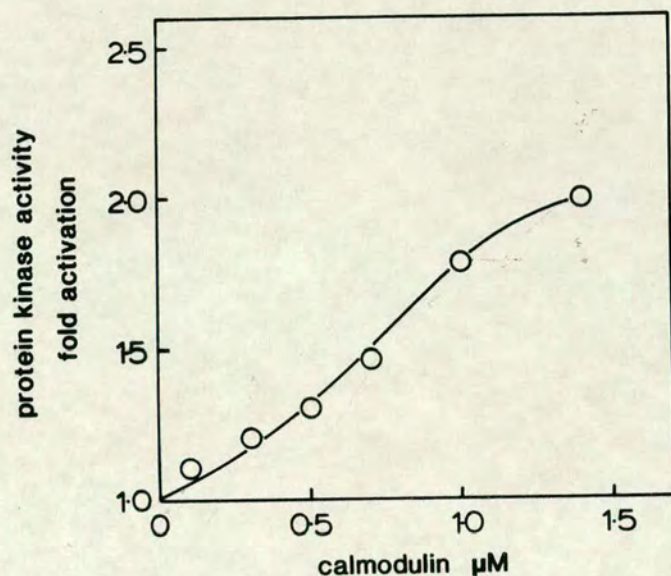
autophosphorylation is visible. The minor band of ~19KDa has also been observed in the acetone solubilised 'plasma membrane enriched' fraction (see Chapter 3). The protein kinase does not phosphorylate the added calmodulin.

It would be reasonable to conclude that the enzyme represented by the ~18KDa protein is that responsible for the calcium activated protein kinase activity observed in the intact membranes. To prove this conclusively would require specific inhibition, possibly by antibodies, of its activity.

Calmodulin Titration of the Electroelulted Protein Kinase

A titration of enzyme activity against calmodulin was necessary to determine an approximate affinity of the protein kinase for this protein. Electroeluted protein kinase from acetone solublised 'total' membrane was assayed with increasing concentrations of calmodulin whilst keeping all other parameters constant. Such protein kinase assays were performed and initial rates estimated by incorporated phosphate after 30 seconds reaction time (see Figure 16). The results are presented in Figure 17.

Figure 17a shows a plot of fold enzyme activation, over the rate in the absence of calmodulin, against calmodulin concentration. It can be seen that ~2 fold activation is obtained at 1.5μM bovine calmodulin, agreeing with the data presented in Figure 16 where apparent 2.2 fold activation was obtained with 3μM calmodulin at the 30 second point. The apparent slight sigmoidal shape of the titration curve would suggest a deviation from 'linear' Michaelis-Menten type kinetics, where a hyperbola would be obtained. Figure 17b, a Lineweaver-Burk plot of the data shown in Figure 17a, illustrates this point. The observation of non linear kinetics would



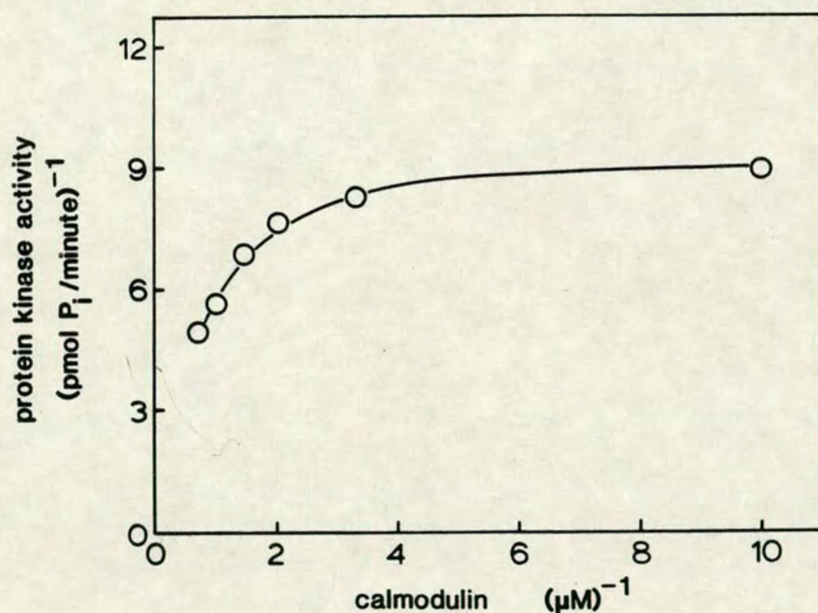


FIGURE 17a.
Calmodulin titration of protein kinase activity in the electroeluted fraction. A 'total' membrane preparation was produced and subjected to acetone solubilisation. After separation by non-denaturing PAGE the autophosphorylating protein kinase containing band was located by comparison with a separate lane labelled using [γ-^{32}P]ATP. The protein kinase containing band was electroeluted from homogenised gel slices. The subsequent eluate was assayed for protein kinase activity using [γ-^{32}P]ATP in the presence of ~100µM free calcium ions over a range of concentrations of bovine calmodulin. Assays were performed at 0°C with 30 second samples as an estimate of initial rate. Data is presented as fold activation of the activity in the absence of calmodulin. Each point represents the average of duplicate determinations.

FIGURE 17b.
A Lineweaver-Burk plot of the data presented in Figure 17a.

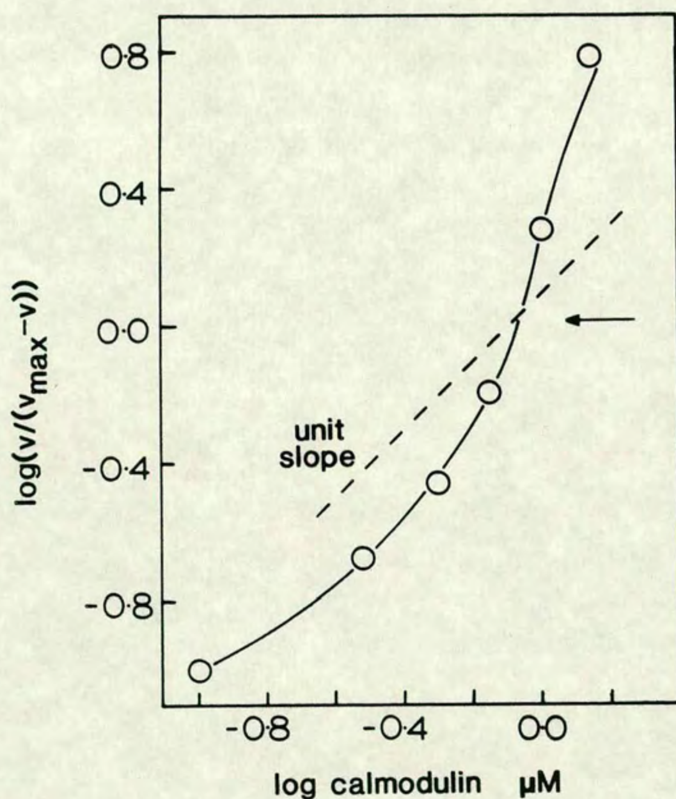


FIGURE 17c.
A Hill plot of the data presented in Figure 17a. With activation in the absence of calmodulin taken as zero the initial rate data has been used to represent V. V_{max} has been taken as the activation obtained with 3µM bovine calmodulin. The arrow indicates the half saturation point and a unit slope is given for comparison.

suggest that some form of cooperativity is occuring with the binding of calmodulin to the protein kinase. Figure 17c, a Hill plot of the data presented in Figure 17a, gives an analysis of this situation. The data is far from ideal, but at half saturation (arrowed) the gradient of the curve is approximately 2. A unit slope, as would be obtained in the absence of cooperativity, is given for comparison. A gradient of greater than unity at half saturation is indicative of positive cooperativity i.e. increasing affinity for calmodulin with calmodulin binding. A tentative suggestion of two calmodulin binding sites in cooperation is also given by the gradient at half saturation. A stoichiometry of 1:1 for calmodulin and a target protein is most frequently found (Klee + Vanaman 1982).

Results of this type should be regarded with caution and the suggestion of the regulatory importance of apparent positive cooperativity would be premature (Engel 1977). A more complete analysis of the kinetics of calmodulin binding awaits further purification of the enzyme.

The apparent final K_m (Michaelis constant) of the enzyme for calmodulin is in the micromolar range. In comparison to other calmodulin dependent enzymes (see introduction) the latter value is rather high, although it must be remembered that bovine calmodulin is being used with an enzyme from pea. However, calmodulin is a highly conserved molecule in evolutionary terms and when isolated from diverse sources shows remarkably similar biochemical properties (Roberts et al. 1986). To date a complete amino acid sequence for pea calmodulin has not been published.

Phosphopeptide Analysis of the Electroeluted Prtein Kinase

Figure 16 showed that the calcium and calmodulin activation of

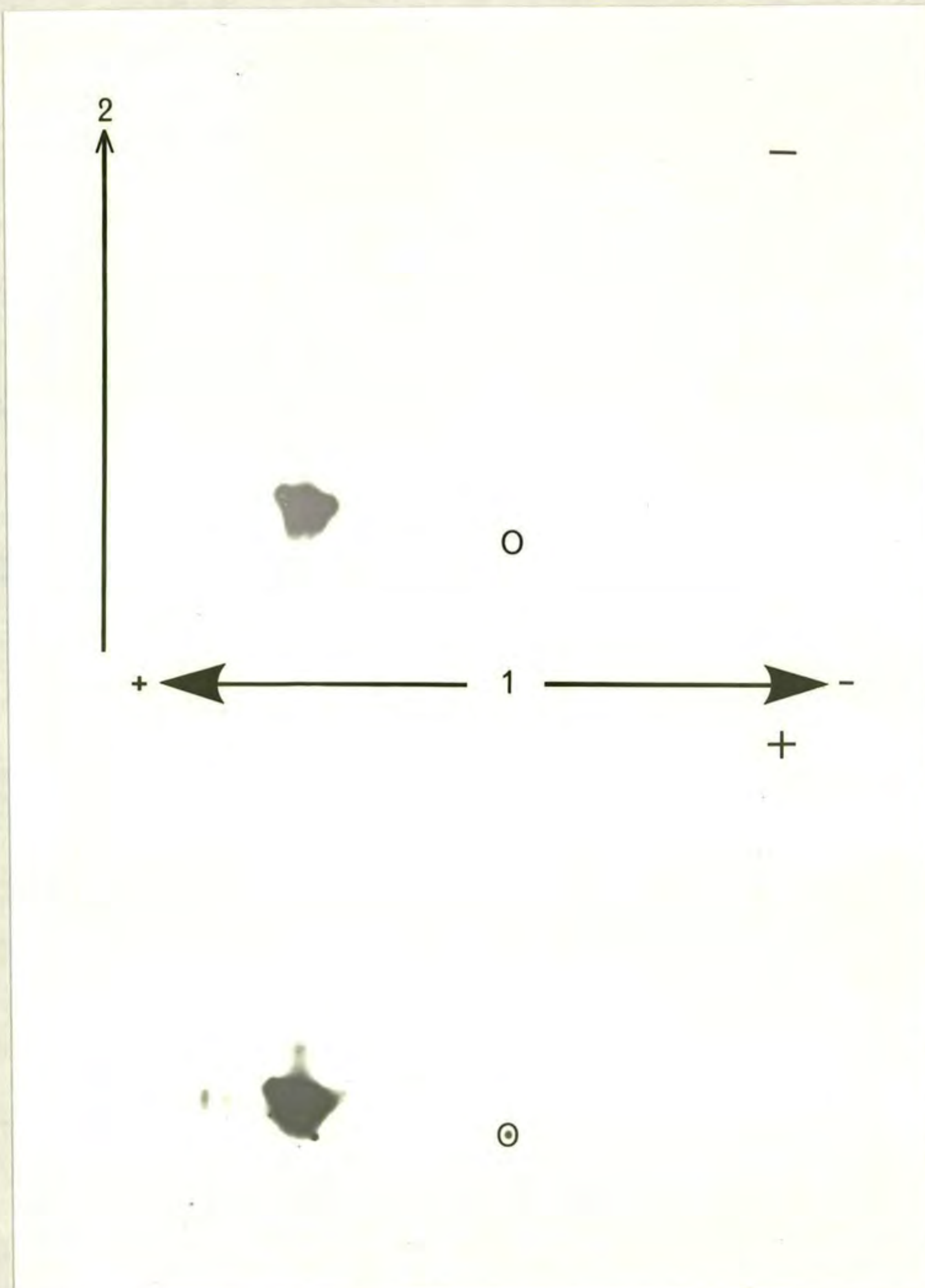


FIGURE 18.
Two dimensional separation of phosphopeptides derived from the autophosphorylated protein kinase. A 'total' membrane preparation was produced and subjected to acetone solubilisation. After separation by non-denaturing PAGE the autophosphorylating protein kinase containing band was located by comparison with a separate lane labelled using [γ-^{32}P]ATP. The protein kinase containing band was electroeluted from homogenised gel slices. The subsequent eluate was labelled using [γ-^{32}P]ATP in the presence of ~100μM free calcium ions, plus or minus 50μg per ml bovine calmodulin, for 30 seconds at 20°C. Protein was TCA precipitated and digested with trypsin overnight. The tryptic digest was then separated in two dimensions on a cellulose thin layer plate. 1st dimension - electrophoresis for 2 hours in 1%(w/v) NH_4HCO_3. 2nd dimension - ascending chromatography in butanol:pyridine:ethanoic acid:dH_2O. The thin layer plates were air dried and autoradiographed. O marks the origin.

the protein kinase autophosphorylation led to a higher equilibrium level of phosphorylation. Whilst it is possible that phosphatases are a component of this reaction, such a result would suggest the phosphorylation of additional sites on the protein in the presence of calcium and calmodulin. To investigate this possibility further the electroeluted protein kinase fraction was labelled using [γ-^{32}P]ATP in the presence of ~100μM free calcium in the presence or absence of 3μM bovine calmodulin. The labelled enzyme produced was then subjected to digestion with trypsin and two dimensional phosphopeptide analysis. The results produced are presented in Figure 18 as autoradiographs of the two dimensional separations. Whilst the increase in phosphorylation in the presence of calcium and calmodulin is apparent, only a single spot is visible for both preparations. Thus it would appear that all sites for phosphorylation are within one peptide produced by tryptic digestion. Attempts to resolve the tryptic digest on urea containing SDS polyacrylamide gels (Burr + Burr 1983) were unsuccessful due to loss of the peptide. It is therefore assumed that the phosphopeptide produced has a very low molecular weight and was lost in the gel staining/destaining. A further analysis of the site of phosphorylation remains to be performed.

Conclusion

For the first time in any preparation of the calcium activated protein kinase a 'significant' activation by exogenous calmodulin has been obtained. It is supposed that the removal of endogenous calmodulin has been achieved during its isolation allowing the demonstration of calmodulin activated autophosphorylation, whether a similar condition exists for the phosphorylation of other substrates in the intact membrane remains to be determined. In a crude membrane

fraction from pea shoots, containing calmodulin, only 1.3 fold activation could be obtained by the addition of 1.2µM bovine calmodulin (Hetherington + Trewavas 1982). Likewise, Putman-Evans _et al._ (1986) only obtained 1.33 fold activation by the addition of 1µM bovine brain calmodulin to a partially purified soluble protein kinase with almost absolute calcium dependence due to the presence of endogenous calmodulin. Activation by the addition of exogenous calmodulin is preferred to the use of inhibitors since the latter is more likely to involve non-specific effects (Roufogalis 1982).

The apparent affinity of the enzyme for bovine calmodulin is rather low and attempted further purification by binding to calmodulin ultrogel has proved unsuccessful. The tendency is to assume that calmodulin is the calcium binding protein involved. The report that the membranes, from which the proten kinase is derived, contain calmodulin (Hetherington + Trewavas 1984a) and the stimulation here by the addition of exogenous calmodulin does not preclude the presence of another calcium binding protein.

Further analysis of the peptide containing the phosphorylation sites is desirable. Other proteases with different site specificities would be useful, although a complete analysis must await amino acid sequencing. The stoichiometry of phosphate incorporation requires further bulk purification of the protein kinase such that amounts of protein can be estimated.

COLUMN CHROMATOGRAPHY ANALYSIS OF THE ACETONE SOLUBILISED 'PLASMA MEMBRANE ENRICHED' FRACTION

Chapter 4 showed that a protein of molecular weight ~18KDa on SDS polyacrylamide gels had protein kinase activity. Since it is not uncommon for proteins to migrate with erroneous apparent molecular weights upon SDS-PAGE (eg Histone H1, see chapter 7, and calmodulin in the presence and absence of calcium (Allan + Trewavas 1985)) a determination of molecular weight by other means was desirable. Gel permeation column chromatography is a suitable method although the assumption of globular proteins is made. Two such procedures were investigated using the acetone solubilised 'plasma membrane enriched' fraction. As described in Chapter 3, this fraction shows little response to added calmodulin whilst activation by ~100µM free calcium alone is apparent (Figure 8a). It is therefore assumed that an endogenous calcium binding protein is present, possibly calmodulin. The separations described below were necessarily performed under non-denaturing conditions and are therefore somewhat equivalent to the non-denaturing PAGE separations described in chapter 4.

Sephadex G100 Column Chromatography of the Acetone Solubilised 'Plasma Membrane Enriched' Fraction

In order to minimise dilution of the small amount of protein present in the preparations a column was constructed in a narrow tube. The column was calibrated using protein standards and the selectivity curve is presented in Figure 19a. Analysis revealed that as much as 250µg of a single protein could be collected in nine fractions. Twenty fractions were collected between the void and total volumes and between 10 and 20µg of the acetone solubilised 'plasma membrane enriched' fraction were loaded for a single run. The column fractions

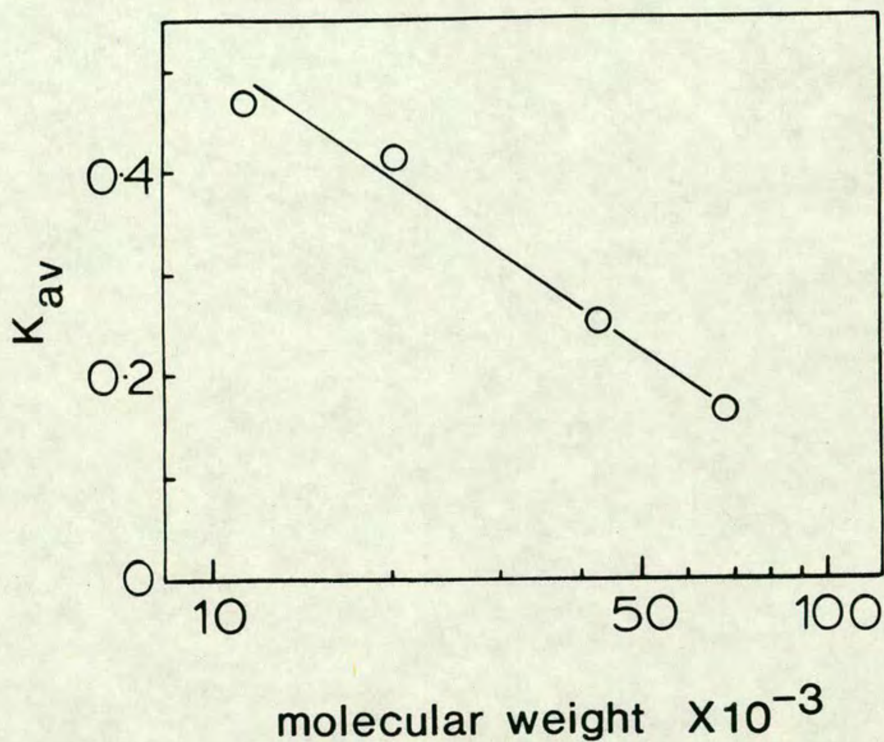


FIGURE 19a.
Selectivity plot for the Sephadex G100 column. The column was calibrated with 250µg loadings of cytochrome c (12.5KDa), soybean trypsin inhibitor (21KDa), ovalbumin (43KDa) and BSA (68KDa). Protein was estimated by absorbance at 280nm, giving the elution volume V_e. Blue dextran and ATP were used to estimate the void (V_o) and total (V_t) volumes respectively. K_{av} is defined as $(V_e - V_o)/(V_t - V_o)$.

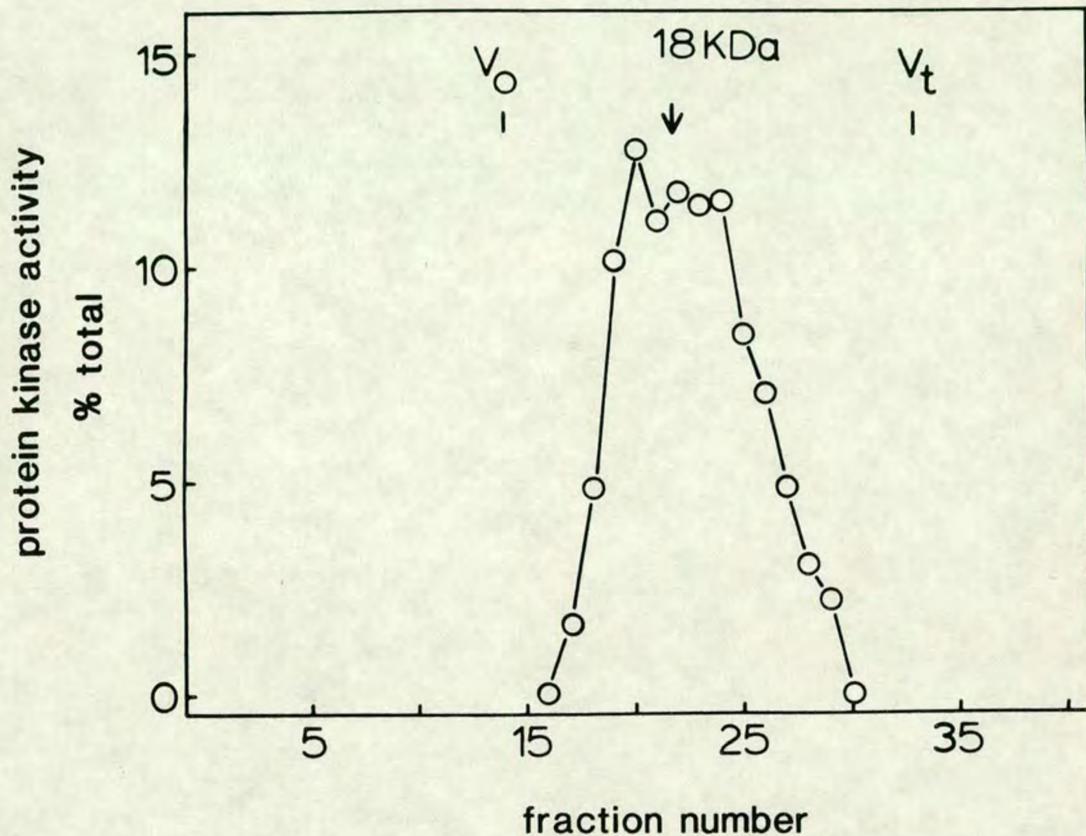


FIGURE 19b.
Elution profile for protein kinase activity, derived from a solubilised membrane preparation, on Sephadex G100. A 'total' membrane preparation was produced and phase partitioned to give a 'plasma membrane enriched' fraction. This membrane fraction was then subjected to acetone solubilisation. Typically, 10 to 20µg of protein from the acetone solubilised 'plasma membrane enriched' fraction were loaded for each run. Protein kinase activity in the column fractions was estimated using [γ-^{32}P]ATP in the presence of ~100µM free calcium ions and 50µg per ml bovine calmodulin. A sample after 30 seconds reaction time at 0°C was taken as an estimate of the activity. V_o, void volume; V_t, total volume. The ~18KDa point, estimated from Figure 19a, is arrowed. Each point represents the average of four separate runs.

were assayed for protein kinase activity, a single point at 30 seconds, in the presence of ~100µM free calcium and 50µg per ml bovine calmodulin. The inclusion of calmodulin is not absolutely necessary, but should ensure maximal activity in all fractions. The elution profile for protein kinase activity, autophosphorylation, is presented in Figure 19b and is the average of four separate runs. The profile contains one peak centred around ~18KDa and therefore confirms the data obtained by SDS-PAGE analysis. However, the peak is broader than desirable and could be composed of more than a single component, free enzyme and enzyme bound to calmodulin could be within its range of molecular weight. In similar separations the column fractions were assayed in the presence of ~100µM free calcium with or without 50µM trifluoperazine (TFP), a calmodulin inhibitor. The only effect of the added TFP was to slightly reduce the overall profile obtained in its absence, and the peak height in the presence of calcium alone was approximately one third of that obtained in the presence of calcium and calmodulin confirming the data of Chapter 5, Figure 15. It would thus appear that in the separation of the acetone solubilised 'plasma membrane enriched' fraction by Sephadex G100 the protein kinase and its calcium binding protein have been separated, suggesting a weak attachment. The precise molecular weight of native pea calmodulin is not known, although its apparent molecular weight after SDS-PAGE is ~16 to ~21KDa depending on the presence and absence of calcium (Allan + Trewavas 1985). Calmodulin would therefore be expected to elute along with the protein kinase in the above column fractions, although dilution of the original sample will have occurred, possibly leading to a decrease in its association with the enzyme. Calmodulin assays on the column fractions have not been performed and therefore the identity of the calcium binding protein in this fraction remains

obscure.

Protein Content of the Protein Kinase Containing Sephadex G100 Column Fractions

As a final analysis of this system the protein kinase containing fractions produced by Sephadex G100 column chromatography were subjected to SDS-PAGE and the component proteins silver stained. A photograph of one such gel is presented in Figure 20. Whilst the proteins present are near the limits of detection it is clear that two major staining bands are present, ~28KDa and ~18KDa, the latter is assumed to be the protein kinase. The distribution of these proteins in the column fractions is in agreement with separation based on their individual molecular weights. However, it was subsequently discovered, whilst using spun columns, that the enzyme adheres to sephadex. In the above separations the location of the peak of protein kinase acitivity around ~18KDa may be fortuitous i.e. an under estimation of the true molecular weight of the protein kinase or a combination of the ~28KDA and ~18KDa proteins.

An attempt was made to separate a pre-labelled acetone solubilised 'plasma membrane enriched' fraction on the Sephadex G100 column and the results are presented in Figure 21. The bulk of the excess [γ-^{32}P]ATP was removed by spinning the preparation through a Bio-Gel P6 desalting gel (Bio-Rad laboratories, Richmond, Cal., USA) column in a bench centrifuge. The only activity eluted from the Sephadex G100 column (Figure 21a) was in the void volume suggesting a molecular weight of greater than or equal to 150KDa, excluding the above mentioned adherence to Sephadex. However, subsequent analysis of this activity by SDS-PAGE revealed a single labelled band at ~18KDa (Figure 21b). It would appear that phosphorylation leads to

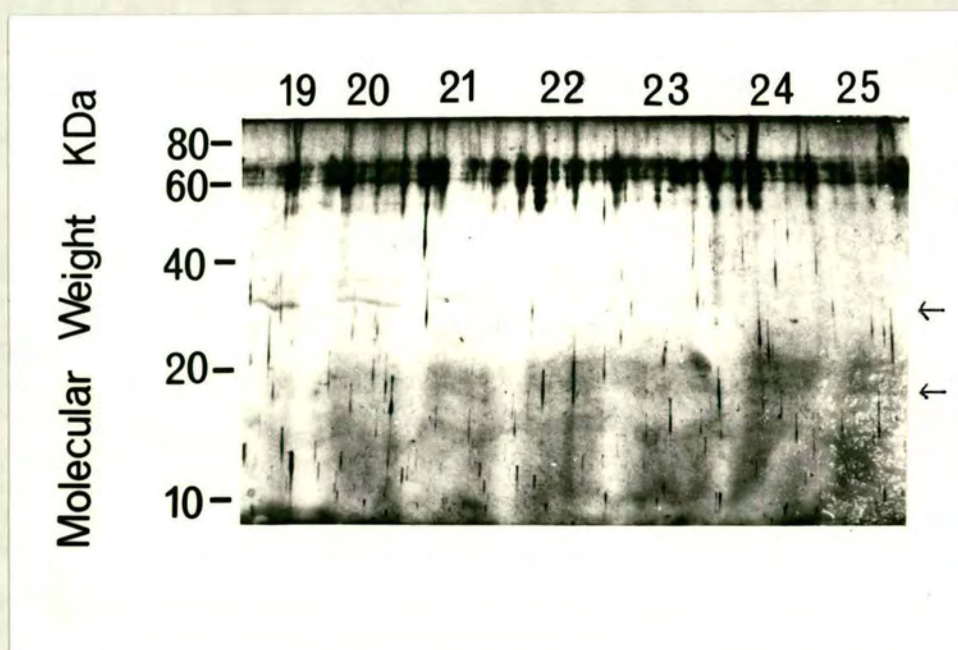


FIGURE 20.
Silver staining of SDS-PAGE separations of the Sephadex G100 column fractions for the acetone solubilised 'plasma membrane enriched' fraction. A 'total' membrane preparation was produced and phase partitioned to give a 'plasma membrane enriched' fraction. This membrane fraction was then subjected to acetone solubilisation and separation on a Sephadex G100 column. Fraction numbers 19 to 25 inclusive of Figure 19b were separated by SDS-PAGE and silver stained. Bands at ~18KDa and ~28KDa are arrowed. The staining bands c.60KDa are artifactual.

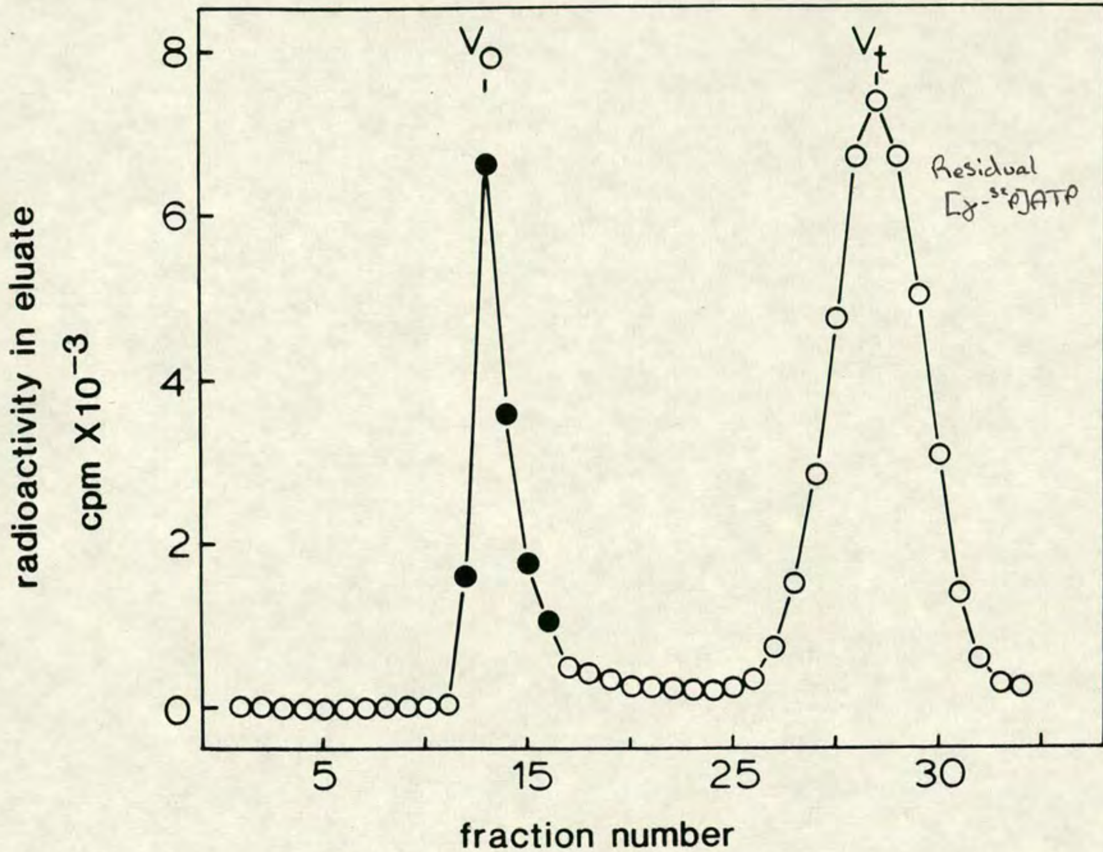


FIGURE 21a.
Elution profile for a prelabelled acetone solubilised 'plasma membrane enriched' fraction separated by Sephadex G100 column chromatography. A 'total' membrane preparation was produced and phase partitioned to give a 'plasma membrane enriched' fraction. This membrane fraction was then subjected to acetone solubilisation and labelled using [γ-^{32}P]ATP in the presence of ~100µM free calcium ions for 30 seconds at 0°C. After removal of excess [γ-^{32}P]ATP in a Bio-Gel P6 spun column the Sephadex G100 separation was performed. Radioactivity in the column fractions was estimated by Cerenkov counting. V_o, void volume; V_t, total volume. Closed symbols indicates those fractions subjected to SDS-PAGE in Figure 21b.

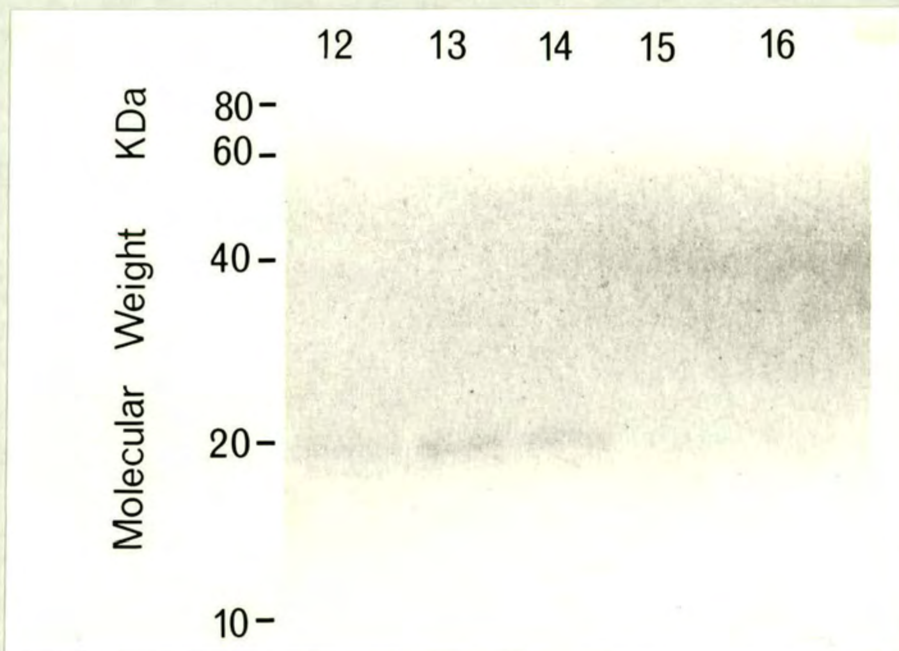


FIGURE 21b
SDS-PAGE analysis of Sephadex G100 column fractions produced by a prelabelled acetone solubilised 'plasma membrane enriched' fraction. The fractions represented by the closed symbols in Figure 21a were separated by SDS-PAGE and the resultant dried down gel autoradiographed.

aggregation of the protein kinase with itself or other proteins. Presumably such aggregations are present in the non-denaturing PAGE separations of acetone solubilised membrane preparations presented in Chapter 4. No further analysis of this observation has been made. This result becomes relevent when considered with the data presented in Chapter 7 where it is shown that phosphorylation of the enzyme leads to a decrease in its protein kinase activity towards histone H1 as an exogenous substrate.

FPLC Separation of the Acetone Solubilised 'Plasma Membrane Enriched' Fraction

Fast Protein Liquid Chromatography (FPLC) can be used for a molecular weight determination and provides excellent resolution when compared to the above Sephadex G100 chromatography. An FPLC system was made available courtesy of Dr.C.J.Leaver, and the results below present an analysis equivalent to part of that above.

Figure 22 gives the elution profiles of protein and protein kinase activity, autophosphorylation, of ~8μg of acetone solubilised 'plasma membrane enriched' fraction separated by FPLC. The very sensitive trace for protein (absorbance at 280nm) shows clearly the complex nature of the preparation. The histogram for protein kinase activity (a single point at 30 seconds), assayed in the presence of ~100μM free calcium and 50μg per ml bovine calmodulin, shows a single major peak, coincident with a peak of protein. However, this major peak of protein kinase activity has eluted from the column with an apparent molecular weight of ~55KDa, not agreeing with the questionable Sephadex G100 data above. Two very minor peaks of protein kinase activity are also present (~22 and ~28KDa).

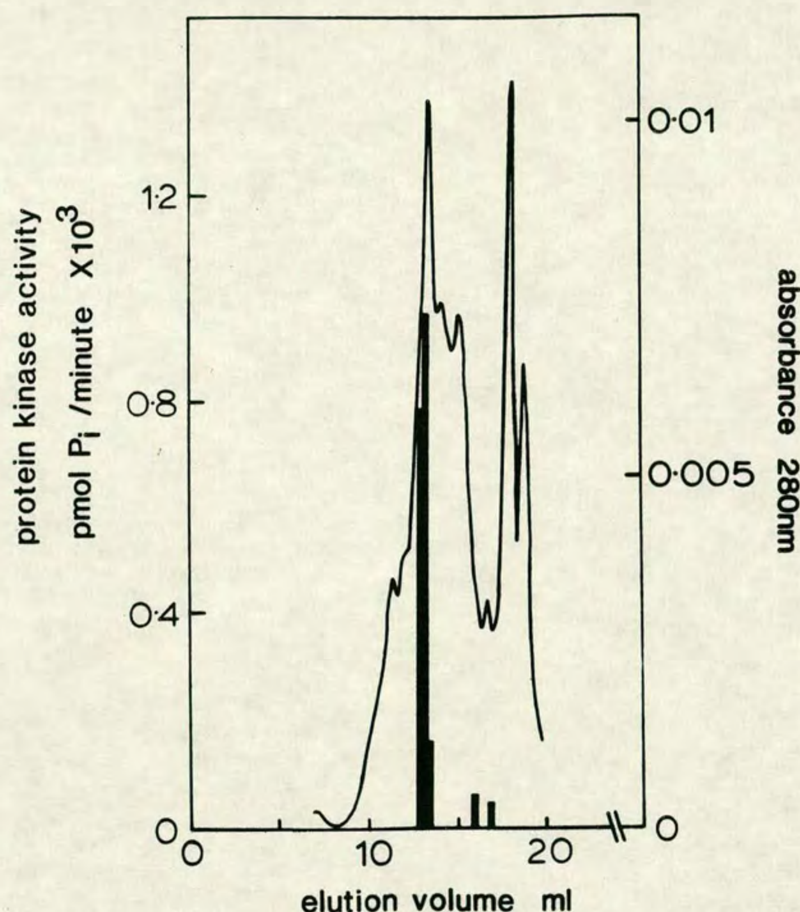


FIGURE 22.
Elution profiles for protein (line trace) and protein kinase activity (histogram) produced by FPLC separation of an acetone solubilised 'plasma membrane enriched' fraction. A 'total' membrane preparation was produced and phase partitioned to give a 'plasma membrane enriched' fraction. This membrane fraction was then subjected to acetone solubilisation and ~8µg of the protein produced separated by FPLC on a Superose 12 column. Column fractions were assayed for protein kinase activity using [γ-^{32}P]ATP in the presence of ~100µM free calcium ions and 50µg per ml bovine calmodulin. Activity was estimated by a single sample after 30 seconds reaction time at 0°C. Protein content of the eluate was monitored as the absorbance at 280nm.

Protein Content and Labelling of the Protein Kinase Containing FPLC Column Fractions

The column fractions containing protein kinase activity were further analysed by SDS-PAGE and autoradiography of the dried down gel. The result for the major peak, fraction number 29, is shown in Figure 23a and for comparison the unfractionated preparation is also shown (see also Figure 8a). As before a labelled band is present of molecular weight ~18KDa, for reasons not known another band of slightly higher molecular weight is also present. A very minor phosphorylated band in approximately this position was sometimes observed in the acetone solubilised 'plasma membrane enriched' fraction and the electroeluted fraction from the acetone solubilised 'total' membrane. Why the phosphorylation of this band should be so enhanced in this preparation is not known.

Finally, the protein composition of the fractions containing protein kinase activity was examined by SDS-PAGE and silver staining. The result for fraction 29 is shown in Figure 23b. Only two major bands are present, ~18 and ~28KDa, as seen in the Sephadex G100 column fractions above. Several minor bands of molecular weight ~55 to ~65KDa are also seen and would be expected to elute in this portion of the separation. It would thus appear that the ~18KDa protein kinase is in association with the ~28KDa band. Whether any such association activates the enzyme remains to be determined, although it can be said that the ~18KDa protein kinase is not totally dependent on assocaition with any such protein from the renaturation data of Chapter 4, Figure 14.

Apart from the usage of FPLC the only experimental difference between the separations shown in Figures 20 and 18b is the buffer composition. For the Sephadex G100 separations PBS (150mM NaCl, 10mM

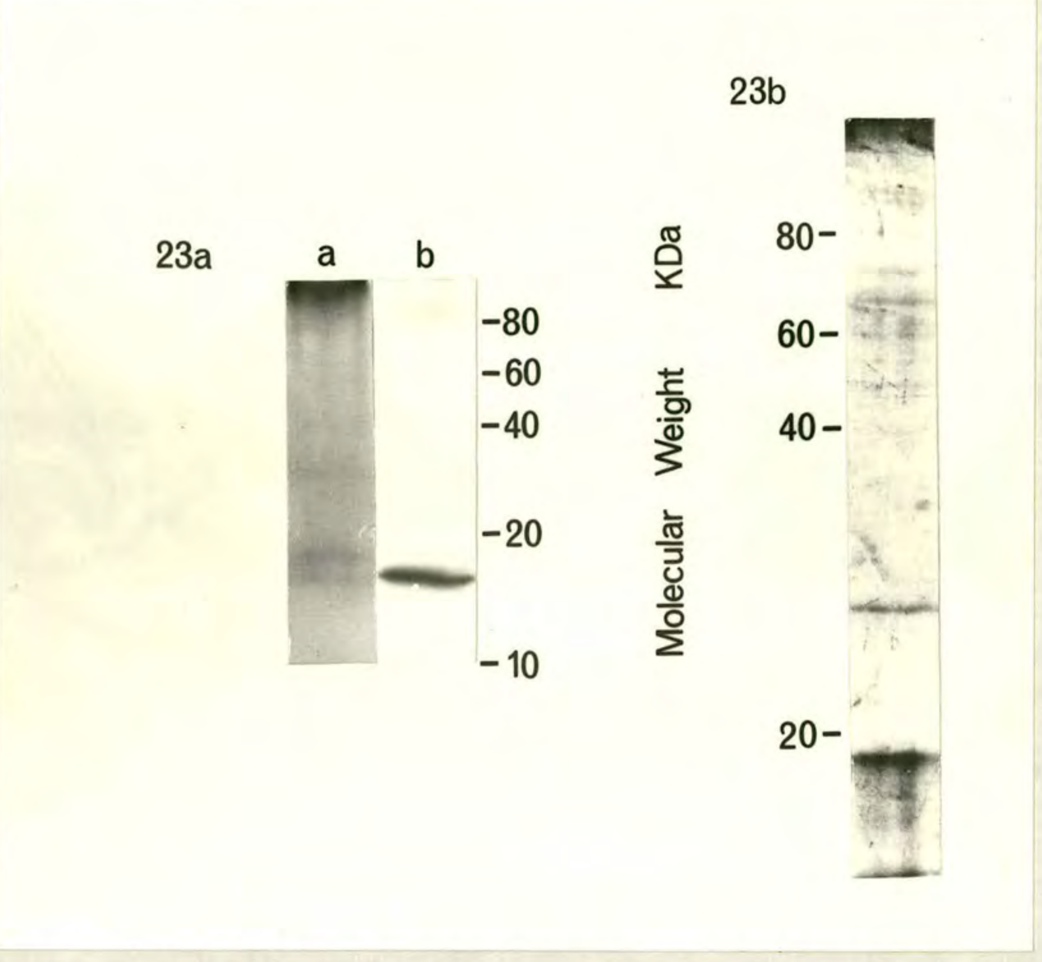


FIGURE 23a.
SDS-PAGE analysis of proteins labelled by a protein kinase containing FPLC fraction produced by the acetone solubilised 'plasma membrane enriched' preparation. Fraction number 29, corresponding to the major peak of protein kinase activity in Figure 22, has been used. Proteins were labelled using [γ-^{32}P]ATP in the presence of ~100μM free calcium ions and 50μg per ml bovine calmodulin for 30 seconds at 0°C. After separation by SDS-PAGE the dried down gel was autoradiographed (lane a). For comparison a similarly treated unfractionated preparation of the acetone solubilised 'plasma membrane enriched' fraction is also shown (lane b).

FIGURE 23b.
Silver staining of an SDS-PAGE separated protein kinase containing FPLC fraction produced by the the acetone solubilised 'plasma membrane enriched' preparation. Fraction number 29, corresponding to the major peak of protein kinase activity in Figure 22, has been separated by SDS-PAGE and proteins silver stained.

sodium phosphate buffer pH7.2) was used and for the FPLC 50mM sodium phosphate buffer pH7.2. It is possible that the higher salt concentration in the Sephadex G100 separations has prevented an association of the protein kinase with another protein.

Conclusion

In conclusion it can be said that the Sephadex G100 data agrees with that obtained by SDS-PAGE although this may be fortuitous. The lack of inhibition by TFP suggests that the ~18KDa protein represents the enzyme isolated from its calcium binding protein.

The observation of an association of the protein kinase with another protein (~28KDa) in FPLC analysis requires further attention, although it may be found to be artifactual. Independent isolation and reconstitution of the two proteins is necessary, and any influence of the degree of autophosphorylation on association investigated. The ~28KDa protein may represent a novel calcium binding protein. A ~28 KDa band is seen to be abundant in both the acetone solubilised 'total' and 'plasma membrane enriched' fractions (see Figures 7 and 8b) and it will be shown in Chapter 8 that antibodies from mice have been raised to this protein.

The reasons for the very much enhanced labelling of another band seen in the FPLC fractions (Figure 23a) is not known, although it is possible that the enzyme has been separated from an inhi bitor present in the whole solubilised 'plasma membrane enriched' fraction. The nature of the two very minor peaks of protein kinase activity in the FPLC separation has not been determined.

Clearly this section of work is very far from complete and the continued use of FPLC analysis on this system is desirable.

AUTOPHOSPHORYLATION AND ITS EFFECTS ON PROTEIN KINASE ACTIVITY

As outlined in the introduction the ability of a protein kinase to autophosphorylated is by no means unusual. However, a clear distinction should be made between inter- or intramolecular autophosphorylation. Intermolecular autophosphorylation is simply the phosphorylation of one enzyme by another, but intramolecular autophosphorylation is the transfer of phosphate from ATP onto the same enzyme molecule or tightly bound subunit. To distinguish between the two it is necessary to investigate the kinetics of labelling in response to enzyme concentration. Intramolecular autophosphorylation would be expected to show saturation type kinetics i.e. the specific activity of the phosphorylation reaction would not be enzyme-concentration dependent (Kuret + Schulman 1985).

In some instances it is known that autophosphorylation has a specific effect on the protein kinse activity (see introduction). Amongst these one group of enzymes is of particular interest, the calcium and calmodulin dependent (MAP2 and type II) protein kinases from brain (Kuret + Schulman 1985; Miller + Kennedy 1986). As members of a large family of homologous brain protein kinases they are calcium and calmodulin dependent and exhibit intramolecular autophosphorylation. The autophosphorylation alters calmodulin binding and reduces the enzyme activity towards exogenous substrates to ~35% of the initial value. The activity of the phosphorylated enzyme is not dependent on calcium or calmodulin.

Evidence has been presented in the previous chapters for calcium and calmodulin activated autophosphorylation of the protein kinase isolated from pea. Whilst the ~18KDa catalytic protein had not been isolated to homogeneity it was considered worthwhile to carry out an analysis of some of the above properties. Since in both the acetone

solubilised 'plasma membrane enriched' fraction (Chapter 3, Figure 8a) and the protein kinase band electroeluted from non-denaturing separations of the acetone solubilised 'total' membrane fraction (Chapter 5, Figure 15) the only labelled band was the enzyme itself these preparations were used.

<u>Enzyme Dilution and an Exogenous Substrate to Demonstrate Intramolecular Autophosphorylation</u>

In order to demonstrate intramolecular autophosphorylation it is necessary to make a comparison between phosphorylation of an exogenous substrate and the enzyme itself. Histone H1 was found to be a suitable substrate for the enzyme (see below and Figure 27). Due to its highly basic nature histone H1 migrates with an erroneously high molecular weight on SDS polyacrylamide gels, ~35KDa as opposed to the 'expected' ~21KDa. Thus, the histone H1 band runs well clear of the protein kinase in an SDS-PAGE separation. To assay the phosphorylation of histone H1 and the enzyme it was necessary to separate such assay mixtures by SDS-PAGE and estimate incorporated activity by counting of gel slices. However, by doing so a dilemma is encountered. To obtain incorporations representative of an initial rate the labelling times had to be short and consequently the counts obtained were low, leading to inaccuracies. Nevertheless, as will be seen below, differences were sufficiently marked so as to alleviate this problem.

Using the electroeluted protein kinase preparation prepared in Chapter 5 the following assays were performed. In the presence of a constant ~100μM free calcium and 50μg per ml bovine calmodulin the enzyme was assayed for autophosphorylation over an eight fold concentration range, reactions were terminated 10 seconds after the

addition of [γ-^{32}P]ATP by the addition of sample buffer (SBX2). Likewise, assays were performed with the inclusion of histone H1 at a starting concentration of 0.8mgml^{-1} and co-diluted with the enzyme. The results of estimation of incorparted activity in bands from SDS polyacrylamide gels are presented in Figure 24. Slices from directly above each labelled band have been used as blanks. Figure 24a shows a plot of specific activity (% control) against protein (excluding histone) and clearly there is a marked difference for the autophosphorylation and the phosphorylation of an exogenous substrate. The phosphorylation of histone H1 exhibits a dependence on enzyme (protein) concentration, although the gradient of this line appears rather steeper than a general doubling of activity with doubling of protein concentration. In marked contrast the autophosphorylation is virtually independent of enzyme (protein) concentration, although the results are less accurate than desirable. The presence of such saturation kinetics is indicative of intramolecular autophosphorylation.

Figure 24b shows a Van't Hoff plot of the data of Figure 24a, and avoids the calculation of relative specific activity and subsequent amplification of errors. Intramolecular autophosphorylation is revealed by the gradient of ~1 for the enzyme phosphorylation and enzyme concentration dependence by the gradient of ~2 for histone H1 phosphorylation.

As an alternative to and as a verification of the above the autophosphorylation of the electroeluted protein kinase fraction from acetone solubilised 'total' membrane was estimated by the extrapolation of initial rates from phosphocellulose strip assay time courses. This may be regarded as a more accurate estimation of initial rates, although it is open to subjective error. The results

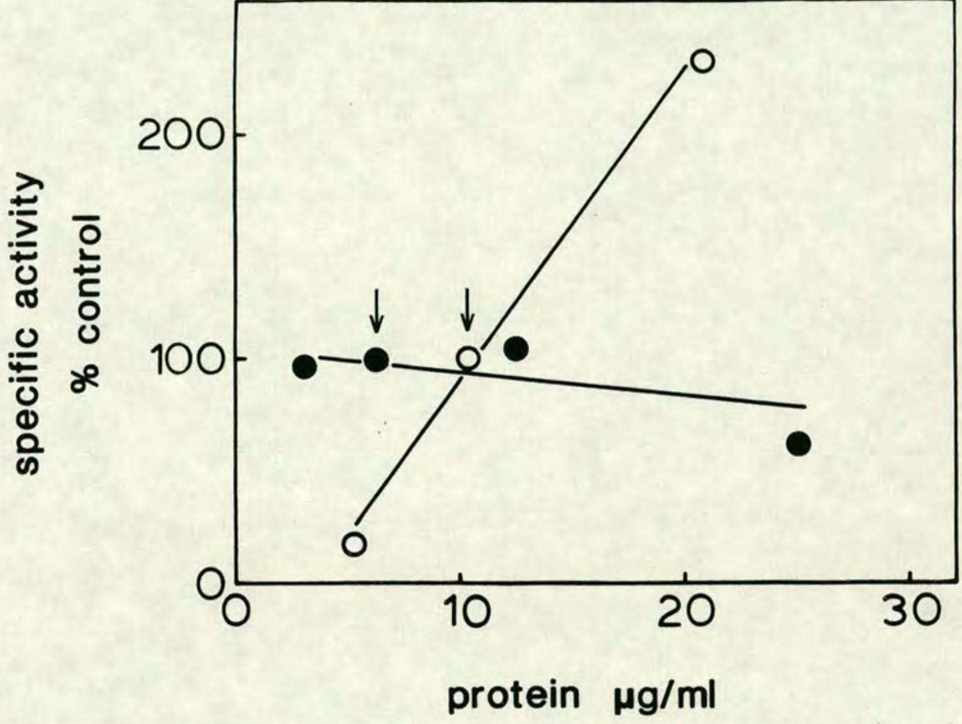


FIGURE 24a.
Graph showing the effect of dilution on protein kinase activity directed towards itself (closed symbols) and histone H1 as an exogenous substrate (open symbols). A 'total' membrane preparation was produced and subjected to acetone solubilisation. After separation by non-denaturing PAGE the autophosphorylating protein kinase containing band was located by comparison with a separate lane labelled using [γ-^{32}P]ATP. The protein kinase containing band was electroeluted from homogenised gel slices. The subsequent eluate was diluted to give protein concentrations as indicated and assayed for protein kinase activity in the presence of a constant amount of [γ-^{32}P]ATP, ~100µM free calcium ions and 50µg per ml bovine calmodulin. Labelling for 10 seconds at 0°C was taken as an estimate of the initial rate and preparations were separated by SDS-PAGE. The appropriate bands were excised and incorporated phosphate estimated by Cerenkov counting. Blanks consisting of gel slices from regions without labelled protein have been subtracted. Data is presented as specific activity normalised about the arrowed points.

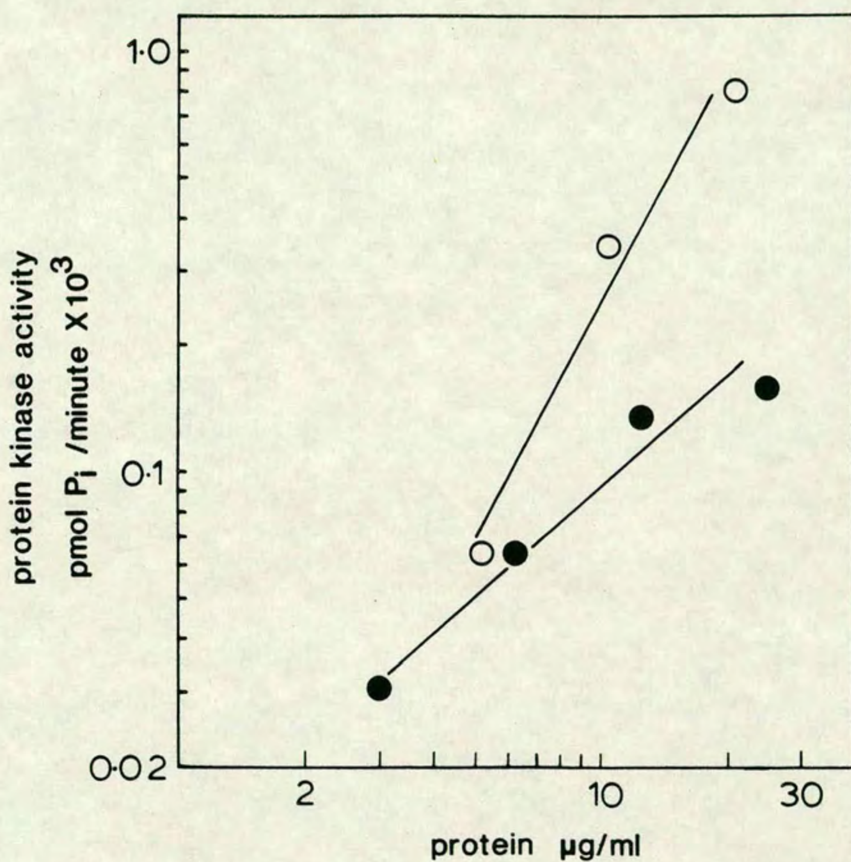


FIGURE 24b.
A presentation of the data of Figure 24a as a Van't Hoff plot. Autophosphorylation (closed symbols) and activity directed towards histone H1 as an exogenous substrate (open symbols) are shown.

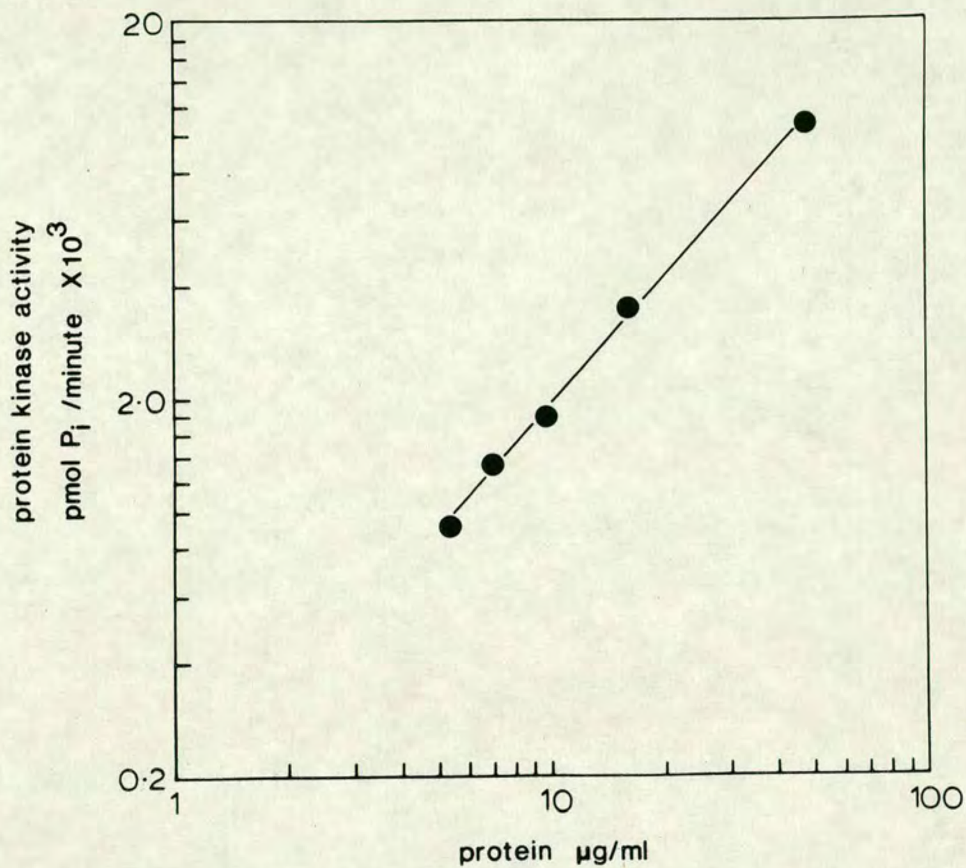


FIGURE 25.
An alternative analysis of the effects of dilution upon the autophosphorylation of the protein kinase. A 'total' membrane preparation was produced and subjected to acetone solubilisation. After separation by non-denaturing PAGE the autophosphorylating protein kinase containing band was located by comparison with a separate lane labelled using [γ-^{32}P]ATP. The protein kinase containing band was electroeluted from homogenised gel slices. The subsequent eluate was diluted to give protein concentrations as indicated and assayed for protein kinase activity in the presence of a constant amount of [γ-^{32}P]ATP, ~100µM free calcium ions and 50µg per ml bovine calmodulin. Initial rates were estimated from the time courses obtained and each point represents an estimate from the average of duplicate time courses. The data is presented as a Van't Hoff plot as in Figure 24b.

of one such experiment, over a nine fold dilution range, are presented in Figure 25 as a Van't Hoff plot. As above, a gradient of ~1 is obtained indicating intramolecular autophosphorylation.

SDS-PAGE of the Autophosphorylation Assay Preparations

It was possible that the results obtained above were the combination of more than a single parameter i.e. to be certain the labelled bands present throughout the wide range of dilutions should be examined. Figure 26 presents an autoradiograph for the dilution of the acetone solubilised 'plasma membrane enriched' fraction over a nine fold range. The only band present throughout is that of the protein kinase at ~18KDa. It should be noted that results similar to those of the above section have also been obtained with the this preparation of the protein kinase.

Histone H1 Phosphorylation and the Effect of Autophosphorylation

To investigate any effect of autophosphorylation on the activity of the enzyme histone H1 was used as an exogenous substrate. An assay was designed such that the enzyme was presented to histone H1 in its isolated state or after phosphorylation. For pre-phosphorylation acetone solubilised 'plasma membrane enriched' fraction was incubated in the presence of ~100μM free calcium and 50μg per ml bovine calmodulin with [γ-^{32}P]ATP. A similar preparation was incubated without ATP. Histone H1 and [γ-^{32}P]ATP added to both assays, twice as much ATP to the non-prelabelled assay to ensure that all final concentrations were equal. The addition of more ATP to the pre-labelled assay is not essential since ATP is present in vast excess. The reactions were terminated and the products examined by SDS-PAGE. Figure 27 (lane a) shows a coomassie blue stained lane for

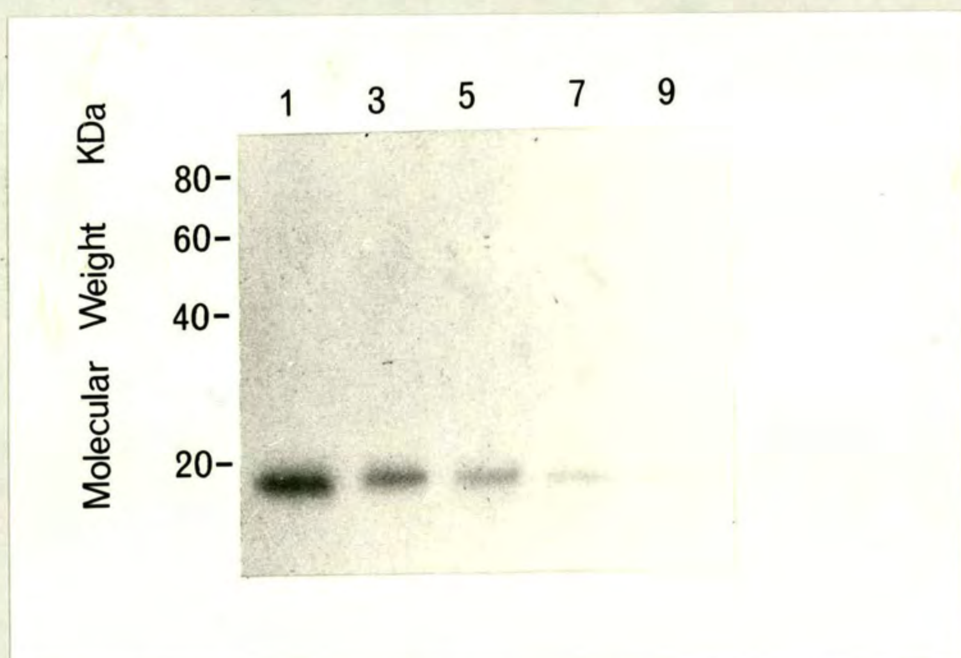


FIGURE 26.
SDS-PAGE analysis of the proteins labelled in dilutions of the acetone solubilised 'plasma membrane enriched' fraction. A 'total' membrane preparation was produced and phase partitioned to give a 'plasma membrane enriched' fraction. This membrane fraction was then subjected to acetone solubilisation. Starting at 50μg protein per ml the acetone solubilised 'plasma membrane enriched' fraction was labelled over a 9 fold dilution range using [γ-^{32}P]ATP in the presence of a constant ~100μM free calcium ions for 30 seconds at 0°C. The preparations were separated by SDS-PAGE and the dried down gel autoradiographed. Numbers above lanes indicate fold dilution.

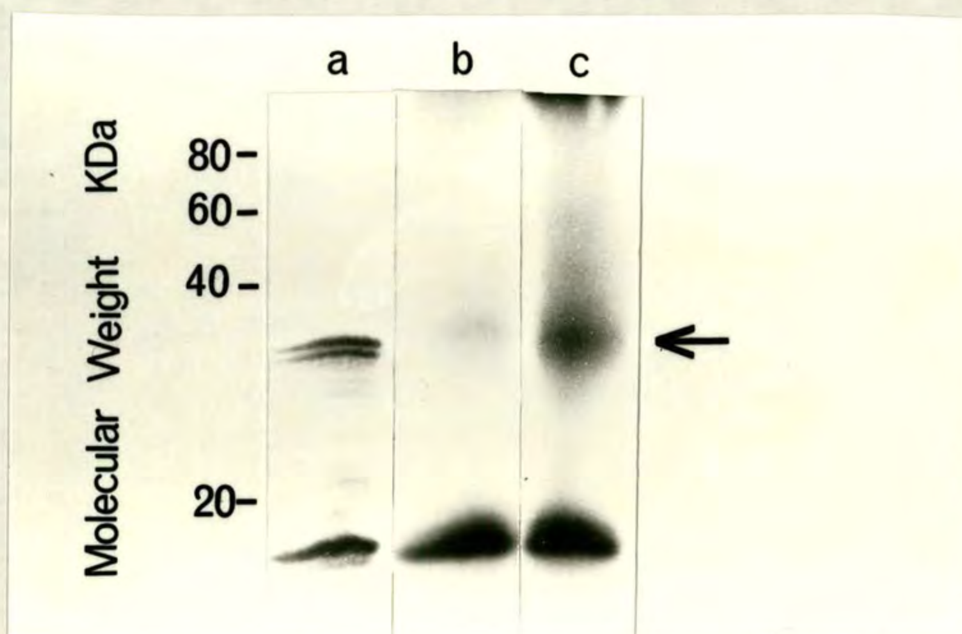


FIGURE 27.
Results illustrating the effect of autophosphorylation on protein kinase activity directed towards histone H1. A 'total' membrane preparation was produced and phase partitioned to give a 'plasma membrane enriched' fraction. This membrane fraction was then subjected to acetone solubilisation. Lanes b and c represent SDS-PAGE separations of the acetone solubilised 'plasma membrane enriched' fraction presented to histone H1 (arrowed) in the presence of ~100µM free calcium ions and 50µg per ml bovine calmodulin. In lane b [γ-^{32}P]ATP was added both before and with the histone. In lane c the same overall amount of [γ-^{32}P]ATP has been added, but none prior to the addition of histone. Thus the protein kinase is presented to the histone in two states of autophosphorylation. Incubations both before and after the addition of 10µg of histone were for 1 minute at 0°C. Lane a shows the coomassie blue staining of lane b.

one of the assays, clearly showing the histone H1 band (~35KDa) and its phosphorylation by the enzyme (lanes b and c). Lane b represents the enzyme which had been pre-phosphorylated prior to the addition of histone and the histone band is less heavily labelled here than in lane c, no pre-phosphorylation. The autophosphorylated protein kinase is present at the same intensity in both lanes and it would thus appear that autophosphorylation of the enzyme leads to a decrease in its activity towards histone H1. Whether the inactivation by autophosphorylation applies to endogenous substrates remains to be determined.

A preliminary attempt has been made to estimate the amount of activity that remains after autophosphorylation. Using a similar protocol to that above the phosphorylation of histone H1 and enzyme were assayed by Cerenkov counting of excised bands from SDS polyacrylamide gels. This method is less accurate than the cellulose disc or phosphocellulose strip methods, but allows the separation of individual proteins. Some results from this type of assay are presented in Figure 28. After a two minute preincubation of the electroeluted protein kinase fraction with or without [γ-^{32}P]ATP the phosphorylation of both enzyme and histone were followed. As would be expected the phosphorylation level of the enzyme which had been prephosphorylated using [γ-^{32}P]ATP remains constant (closed circles). However, the phosphorylation of the enzyme which had not been prephosphorylated (closed triangles) also appears to be constant. Autophosphorylation of the enzyme in this preparation under these conditions is clearly very rapid, unlike the phosphocellulose strip data presented in Figure 16. Nevertheless, the effect of autophosphorylation on histone H1 phosphorylation is readily apparent. After preincubation of the enzyme in the presence of [γ-^{32}P]ATP the

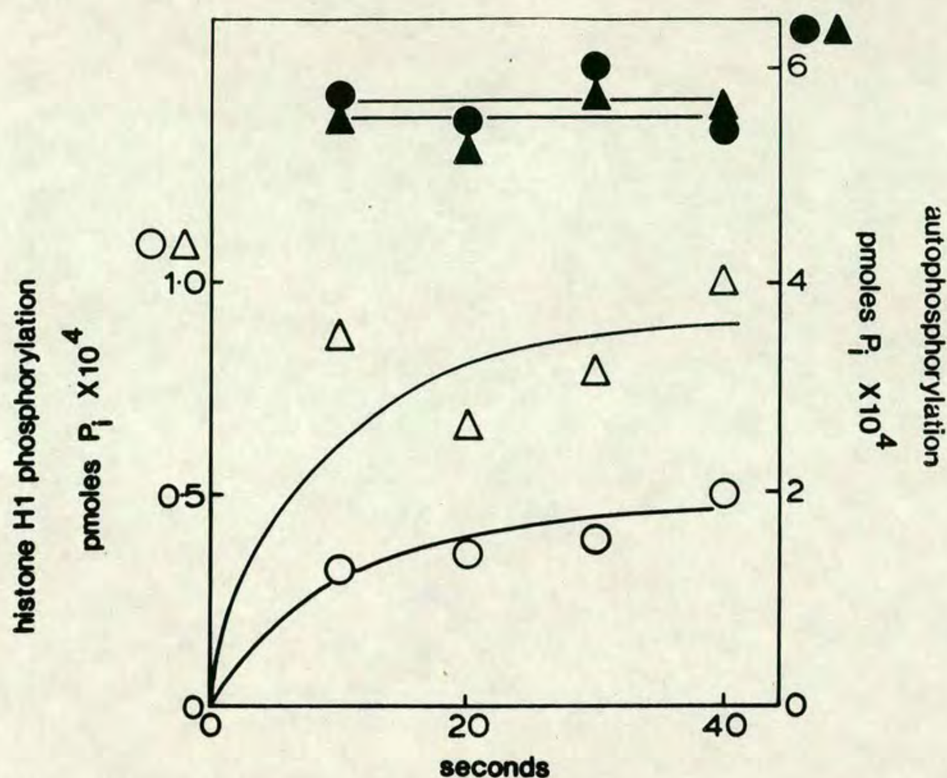


FIGURE 28.
An estimation of the degree of autophosphorylation induced inhibition of protein kinase activity directed towards histone H1. A 'total' membrane preparation was produced and subjected to acetone solubilisation. After separation by non-denaturing PAGE the autophosphorylating protein kinase containing band was located by comparison with a separate lane labelled using [γ-^{32}P]ATP. The protein kinase containing band was electroeluted from homogenised gel slices. The subsequent eluate was used in the following assays. In a similar fashion to the results shown in Figure 27 the protein kinase was presented to histone H1 in two relative states of autophosphorylation. The elecroeluted protein kinase was preincubated for 2 minutes at 0°C in the presence of ~100μM free calcium ions and 50μg per ml bovine calmodulin with (circles) or without (triangles) [γ-^{32}P]ATP. After the addition of more [γ-^{32}P]ATP, such that the final amount was equal in both assays, 10μg of histone H1 was added. Assays were stopped at the appropriate times and separated by SDS-PAGE. Both the protein kinase (closed symbols) and histone H1 (open symbols) bands were excised and incorporated radioactivity estimated by Cerenkov counting. Zero time blanks were obtained by stopping the reactions at the end of the pre-incubation period and before the addition of histone and [γ-^{32}P]ATP.

phosphorylation of histone H1 (Figure 28, open circles) is relatively slow when compared to the unphosphorylated enzyme rate (open triangles). Determination of an initial rate of histone H1 phosphorylation by the relatively unphosphorylated enzyme is difficult due to the inaccuracy of the data and in that a rate equivalent to that of the phosphorylated enzyme is soon reached.

This apparent property of the enzyme requires much further investigation, both in terms of the number of phosphates required and the calcium/calmodulin dependence of the residual activity. Miller and Kennedy (1986) present a complete analysis for a brain derived protein kinase with very similar properties to the enzyme isolated here. Further purification of the enzyme is required before such an analysis can be made.

Conclusion

An analysis of autophosphorylation has revealed it to be intramolecular in nature, although a small component of intermolecular activity cannot be ruled out entirely. Whether autophosphorylation is a significant portion of the enzyme's activity when in the intact membrane is not known, although its labelling in 'plasma membrane enriched' fractions would suggest this to be the case (Chapter 2, Figure 5a).

Autophosphorylation is seen to reduce the activity of the enzyme towards an exogenous substrate, histone H1, although the calcium/calmodulin dependence of the residual activity has not been determined. The variation in calcium/calmodulin dependence of the acetone solubilised 'total' membrane fraction could be explained in terms of differing degrees of autophosphorylation upon isolation if this residual activity were found to be independent of

calcium/calmodulin i.e.calcium dependence of the phosphorylation of other substrates reflects the autophosphorylation state of the enzyme. The rapid autophosphorylation found here by the counting of gel slices does not compare well with the phosphocellulose strip data presented in Figure 16. It is possible that the latter method is detecting the phosphorylation of minor components not seen in an SDS-PAGE type analysis. However, rapid autophosphorylation may be a particular property of this batch of enzyme. Ten second reaction times were used earlier in this chapter for the demonstration of intramolecular autophosphorylation since it is the earliest possible time point after the addition and mixing of [γ-^{32}P]ATP.

To further analyse the autophosphorylation it is clear that the reaction will have to be retarded, possibly by a reduction in the magnesium content of buffers as described by Miller and Kennedy (1986).

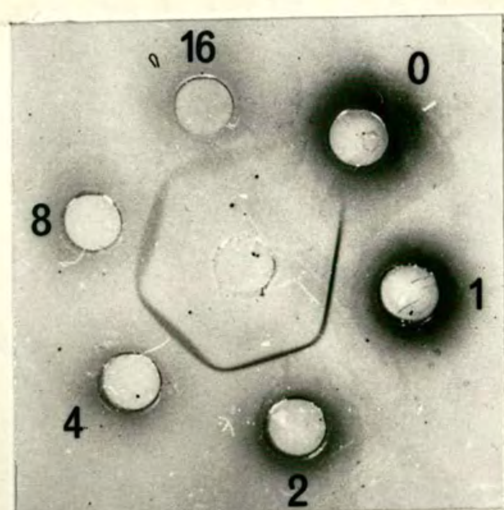
PRODUCTION OF ANTIBODIES AGAINST THE ACETONE SOLUBILISED 'PLASMA MEMBRANE ENRICHED' FRACTION

An antibody preparation to the acetone solubilised 'plasma membrane enriched' fraction has been produced in mice (thanks are due to Dr.Ansell of the Zoology Department for performing the injections and bleedings). Since the acetone solubilised 'plasma membrane enriched' fraction was available in such small amounts (~15µg per 100g fresh weight of tissue) the mice were primed with the acetone solubilised 'total' membrane preparation and later injections of the former preparation were used to potentiate the titre for specific proteins. The primary objective was to raise antibodies to the ~18KDa protein kinase, described in earlier chapters, and isolate these from the whole population by the affinty methods of Smith and Fisher (1984). These antibodies could then, possibly, be used to specifically inhibit the activity of the protein kinase in membrane preparations to assess any involvement in properties such as calcium transport. Sub-cellular distribution and the sidedness of position using right side out, sealed, plasma membrane vesicles from phase partitioning (Larsson et al. 1984) could also be determined (Chan + Schatz 1979).

The results below present an assessment of the final serum produced in this investigation.

Double Diffusion Assays to Estimate Titre

As an initial characterization it was necessary to estimate the titre and check the specificity of the crude serum produced. Double diffusion assays over the range X1 to X1/1024 dilution for the crude serum against an acetone solubilised 'plasma membrane enriched' preparation are presented in Figure 29. Importantly, no detectable



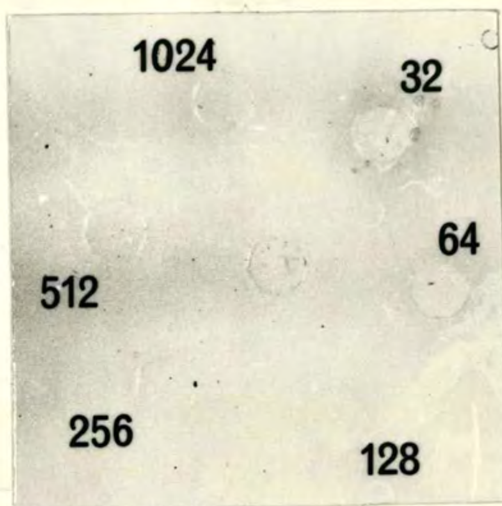


FIGURE 29.
Double diffusion assays of a crude serum produced in mice against an acetone solubilised protein kinase preparation. A 'total' membrane preparation was produced and phase partitioned to give a 'plasma membrane enriched' fraction. This membrane fraction was then subjected to acetone solubilisation, the production of antibodies in mice had been potentiated against this same preparation. The equivalent of 7µg of protein from the acetone solubilised 'plasma membrane enriched' fraction were added to each centre well. Numbers at the outer wells refer to the fold dilution of the serum applied, zero indicates 'normal' mouse serum. After development of precipitin lines overnight the plates were washed and stained with coomassie blue.

precipitation of protein has occurred with the 'normal' mouse serum. From the coomassie blue staining of the precipitin lines it was estimated that a minimum of 1.6µl of crude serum was required to precipitate 1µg of the acetone solubilised 'plasma membrane enriched' fraction. It now remained to be seen which of the proteins present in the solubilised membrane preparation were involved.

EITB Analysis of the Crude Serum

Enzyme linked immunoelectro transfer blotting (EITB) involves the separation of assay protein by SDS-PAGE, blotting to nitrocellulose, tagging of proteins with test antibodies and then development of antibody attachment using a second antibody with coupled enzyme. The reaction of antibodies in the crude serum with specific proteins can thus be visualized. The second antibody used in this case was goat-anti-mouse horse radish peroxidase coupled, as supplied in the Bio Rad kit. The results of such an analysis using a concentrated acetone solubilised 'plasma membrane enriched' preparation are presented in Figure 30. Blotting of a separate lane of the SDS-PAGE separated acetone solubilised 'plasma membrane enriched' fraction and subsequent staining of protein with coomassie blue enabled the correct alignment of reactive bands in the EITB process, lane a of Figure 30 shows such a coomassie blue stained blot. Lane b of Figure 30 shows that there was no cross reactivity of the second antibody with proteins in the preparation, since first antibody was missed out in this case, and lane c shows the complete pattern for the acetone solubilised 'plasma membrane enriched' fraction. Clearly, the crude serum contains antibodies to several of the proteins in this preparation including the ~28KDa band discussed in Chapter 6. However, there appears to be no activity towards the protein kinase

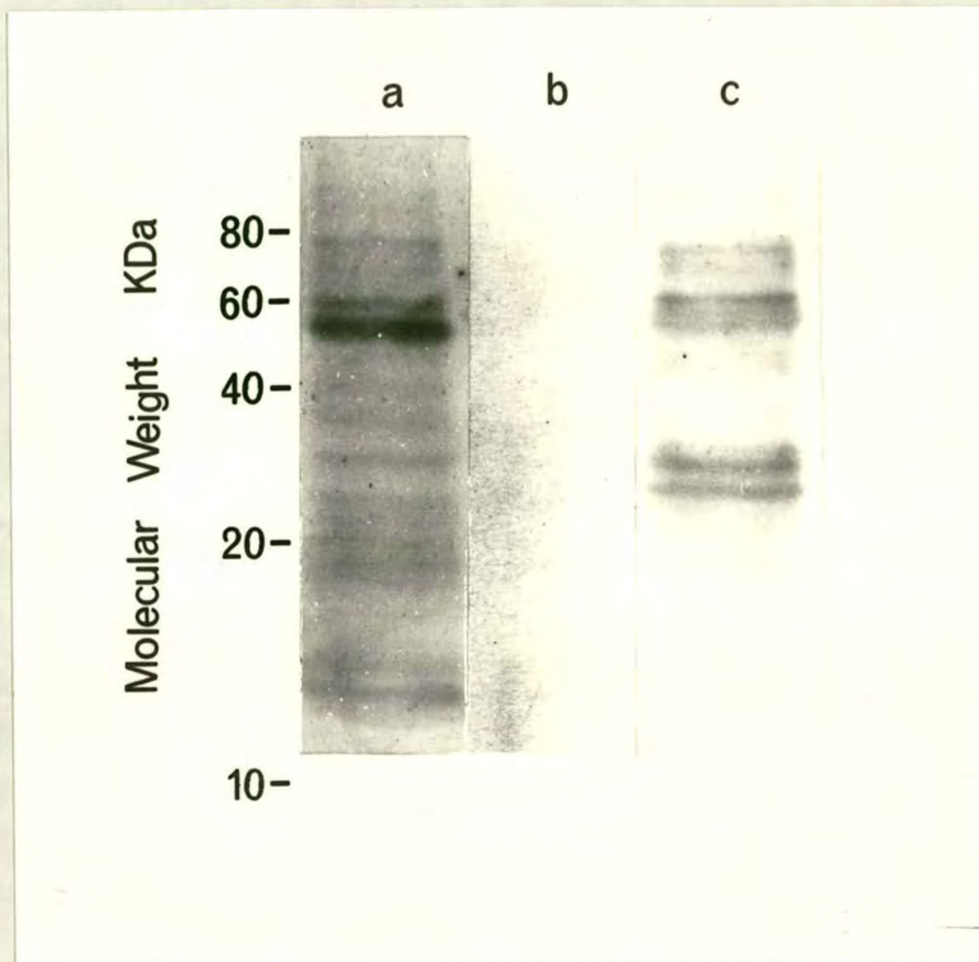


FIGURE 30.
Enzyme linked immunoelectro transfer blotting (EITB) analysis of a crude serum produced in mice against a solubilised membrane preparation. A 'total' membrane preparation was produced and phase partitioned to give a 'plasma membrane enriched' fraction. This membrane fraction was then subjected to acetone solubilisation, antibodies in mice had been potentiated against this same preparation. After separation by SDS-PAGE and blotting to nitrocellulose the proteins were treated in three separate ways: Lane a - coomassie blue stain of the blotted proteins. Lane b - EITB analysis using only the second (Goat-anti-mouse peroxidase linked) antibody. Lane c - EITB analysis using both first (as described above) and second antibodies. After 'development' of peroxidase activity in lanes b and c the nitrocellulose was photographed wet.

band of ~18KDa.

Similar results have been obtained for the acetone solubilised 'total' membrane, 'plasma membrane enriched' fraction and the electroeluted protein kinase fraction. It can therefore be concluded that the antibody titre for the protein kinase is below detectable levels. Under ideal conditions the above method is capable of detecting as little as 100 picograms of a single antigen on nitrocellulose.

Conclusion

The crude serum produced in mice, which has been potentiated towards the acetone solubilised 'plasma membrane enriched' fraction, is clearly a very poor source of antibodies towards the protein kinase. The protein kinase is only a minor component of the above preparation (see Figure 8b) and may well be a 'poor antigen' in mice. If another attempt is to be made to raise antibodies directed towards the protein kinase then the purified protein will have to be used and the ~18KDa regions from SDS-PAGE separations of 'total' membrane may be a suitable source.

GENERAL DISCUSSION

The discussion is composed of two parts. Firstly, a criticism of methods and conclusions. Secondly, a consideration of the knowledge gained concerning protein kinase activity in pea bud membranes over the last two and a half years.

The first major point of criticism is that no *in vivo* labelling of proteins using ^{32}P has been performed. Therefore, it cannot be said with certainty that the reactions observed actually occur in the intact cell. The isolation of the enzyme 'nearest' to the intact cell is probably best represented by the 'plasma membrane enriched' fraction produced by phase partitioning. It is also possible that *in vivo* labelling would have revealed the presence of tyrosine phosphate, this would then be assumed to be the product of tyrosine kinase activity. The use of more than a single peptide substrate as an assay for tyrosine kinase activity is desirable. Random sequence peptides consisting of known amino acids would have been particularly useful since they would present any possible tyrosine kinase with a wide range of sequences surrounding phosphorylation sites.

The phase partitioning method employed, whilst specifically designed for general usage, is not necessarily optimal for the membranes isolated here. A complete analysis covering a range of polymer concentrations, salt types, salt concentrations and pHs should ideally be performed. It is therefore possible that the 'plasma membrane enriched' fraction produced here is not optimal in terms of recovery and purity. Plasma membrane markers, other than those described in the text, are available although all are questioned. The binding of Naphthylphthalamic acid (NPA) to membranes and Phosphotungstic acid-chromic acid (PTAC) staining have been reported

to be useful by other workers. Whilst using the system of phase partitioning designed for general use the repetition of batch type separations may well have yielded a plasma membrane fraction with a higher degree of purity. However, higher purity would be obtained at the expense of recovery.

The method of acetone solubilisation (i.e. precipitation of membrane proteins in acetone, resuspension in an aqueous buffer and centrifugation) is clearly rather drastic and slightly variable. Despite attempts to optimize the process the variability still remained. However, the use of detergents to remove the protein kinase activity from the membrane had already proven to be fruitless.

One major problem is that even the purest preparations of the protein kinase still contain many other proteins. Consequently, data provided by the calmodulin titration of the enzyme should be regarded with some caution. Whilst activation by calmodulin is apparent the precise affinity of the protein kinase for calmodulin is open to question. Other proteins present in the preparation are potentially capable of influencing calmodulin binding to the enzyme. The suggestion of positive cooperativity requires much further investigation, again using more pure preparations of the enzyme.

A further point regarding activation by calmodulin is presented by the experiments in which activation of the enzyme is obtained by calcium alone. In these circumstances it has been assumed that calmodulin, or another calcium binding protein, is present. However, no estimations of calmodulin content have been made for any preparation and the identity of the 'natural' calcium binding protein remains obscure. Whilst calmodulin is known to be present in the 'total' membrane preparation (Hetherington + Trewavas 1984a) it need not be directly involved in the calcium activation of the protein

kinase described here.

The demonstration of intramolecular autophosphorylation is also subject to criticism due to the presence of other proteins in the enzyme preparation. The apparent slight increase in activity upon dilution would suggest the presence of some form of inhibitory action (see Fig. 24a).

No protease inhibitors have been employed during the isolation of the protein kinase and it is possible that the active ~18KDa protein represents a fragment of the whole enzyme.

No experiments have been performed to establish any phospholipid dependence, as might be expected for an enzyme derived from membranes. It is assumed that the enzyme is bound to the cytoplasmic face of the membrane, allowing control by an increase in cytoplasmic free calcium ions. No data is presented to support this assumption.

As a final point of criticism it should be said that no absolute identification of stained and labelled bands on polyacrylamide gels has been made. It is possible that bands of similar molecular weight do not represent the same protein in all instances. The use of antibodies or peptide mapping of individual bands would resolve this issue.

As a preliminary to the discussion of the properties of the enzyme, and a comparison to other protein kinases, a brief summary of knowledge to date will be given.

Protein kinase activity activated by micromolar concentrations of calcium ions is present in membranes isolated from dark grown peas. A majority of this activity is located in the plasma membrane and a protein kinase has been isolated from this source. Partially purified preparations of the enzyme solubilised from the plasma membrane

demonstrate activation by calcium and calmodulin. The enzyme molecule has a molecular weight of ~18KDa and shows an ability to autophosphorylate. It is for this autophosphorylation that an activation by calcium and calmodulin has been demonstrated. Calmodulin activation shows positive cooperativity with two calmodulin binding sites and half maximal activation occurs in the micromolar range. The autophosphorylation appears to occur on an intra-, as opposed to inter-, molecular basis i.e. phosphorylation occurs on the same catalytic unit. The phosphorylation occurs primarily on serine residues that are located in a small peptide fragment(s) produced by tryptic digestion. Upon autophosphorylation the activity of the enzyme towards an exogenous substrate, histone H1, decreases by more than 50%. However, it is not known whether this residual activity is activated by calcium and calmodulin. Autophosphorylation appears to cause aggregation of the enzyme with itself and possibly other proteins.

The knowledge that the enzyme was autophosphorylating enabled a clearer understanding of many observations. For instance, a membrane preparation incubated with ATP would continue to phosphorylate for considerably longer than a solubilised membrane preparation under identical conditions. For the solubilised membrane preparations the time course of phosphate incorporation was characteristically short and ATP was not limiting. It is possible that the rapid autophosphorylation inactivates the enzyme activity directed towards potential substrates present in these preparations.

The only comparisons that can be made to other plant protein kinases are to soluble, not membrane associated, enzymes. Polya and Davies (1982) and Polya and Micucci (1984) have partially purified two calcium and calmodulin activated protein kinases from wheat. The

latter enzyme has an apparent molecular weight of ~86KDa. Putnam-Evans et al. (1986) have isolated a calcium and calmodulin activated protein kinase from soybean, with an apparent molecular weight of ~65KDa on Sephadex G-100 column chromatography. The enzyme of Polya and Micucci (1982) shows half maximal activation with calmodulin at 0.3 micromolar, comparing favourably with the data obtained here, whilst that of Putnam-Evans (1983) showed only slight activation in the presence of one micromolar calmodulin. No suggestion of any cooperativity between more than one calmodulin binding site has been made by any of the above authors.

Brummer and Parish (1983) attempted to identify specific proteins associated with specific membranes from corn. One of the proteins that they found to be a useful 'marker' for the plasma membrane had a molecular weight of 20KDa. This protein was phosphorylated in vivo and thus appears similar to the protein kinase described here.

The enzyme of Miller and Kennedy (1986) from rat brain shows similarity to the enzyme described here in a number of ways. Whilst the enzyme consists of multimers of ~50KDa and ~60KDa subunits, unlike the ~18KDa enzyme from pea, its other features are worth discussing. Each subunit has protein kinase activity and shows intramolecular autophosphorylation (Kuret + Schulman 1985). Upon autophosphorylation the activity of the enzyme towards an exogenous substrate decreases by ~65% and the residual activity is independent of calcium and calmodulin. The enzyme also shows positive cooperativity in activation of autophosphorylation by calmodulin (LeVine et al. 1986). Whilst the precise function of this enzyme is not known it is considered to act as a calcium triggered molecular switch, giving a prolonged response beyond that of the initial calcium signal (Miller + Kennedy 1986). Could the protein kinase described

here have a similar function in plants? If this is the case then the results presented in this thesis are highly significant. The process of autophosphorylation of a protein kinase (albeit intermolecular) has been proposed to be the basis for memory storage in the brain (Lisman 1985).

It is worthwhile considering how the enzyme identified here could act as a molecular switch. However, much needs to be determined before any one mechanism can be chosen. The autophosphorylation of the enzyme clearly inhibits its activity, but is so rapid that if this were the case in the intact plant then phosphorylation of other substrates prior to inactivation would have to be equally rapid. It is possible that sustained phosphorylation of a different sub-set of proteins could be brought about by the autophosphorylated kinase. An attempt could be made to analyse the kinetics of labelling for individual proteins, possibly in a 'plasma membrane enriched' fraction. Those showing very rapid initial phosphorylation would be more likely 'early' substrates for the protein kinase. However, the presence of other protein kinases would influence such data. Likewise, and as observed in results already obtained, some proteins exhibit a lack of calcium dependence in their phosphorylation (see Fig.7). Assuming that all phosphorylations in such a preparation are brought about by the kinase described here, then a lack of calcium and calmodulin dependence after autophosphorylation would explain such observations.

Evidence also suggests that the enzyme aggregates after autophosphorylation, although this may not occur in the intact membrane. Would the removal of phosphate lead to a dissociation of the multimers? The use of a suitable phosphatase and column chromatography would help to resolve this question. It is also

possible that the protein kinase leaves the membrane upon autophosphorylation, giving it access to a new set of substrates and possibly enzymes to regulate. The simple treatment of membrane preparations with or without ATP, spinning down the membranes and assaying the supernatant for protein kinase activity would be a suitable initial experiment in this respect.

A need to isolate phospho and dephospho forms of the enzyme exists. It has already been speculated in chapter 4 that this may be occurring in nondenaturing gel electrophoresis separations and this requires further investigation. It may also be possible to separate the two forms of the enzyme by SDS-PAGE as described by Yamauchi and Fujisawa (1985). An alternative approach would be to dephosphorylate the enzyme with a suitable phosphatase.

An analysis equivalent to that of Miller and Kennedy (1986) is also necessary to demonstrate a molecular switch. They were able to determine the number of phosphates required for a conversion of their protein kinase from one from to the other. Such an analysis must await further bulk purification of the enzyme.

Conclusions concerning a role for the ~18KDa protein kinase in signal transduction can only be tentative. However, the following situation can be envisaged. Upon stimulation, the nature of which remains obscure, the plant cell opens calcium channels in the plasma membrane. Calcium ions enter the cytoplasm giving a localised rise in intracellular concentration. Calcium ions bind to and activate calmodulin which then binds to the membrane associated protein kinase. The protein kinase phosphorylates itself and other substrates of unknown identity. Autophosphorylation reduces the activity of the protein kinase. Calcium pumps in the plasma membrane return the

cytoplasmic calcium concentration to its 'resting' state. The calcium-calmodulin enzyme complex dissociates. If the residual activity of the protein kinase were independent of calcium and calmodulin then its activity would continue until it was degraded or dephosphorylated.

What is the nature of the primary stimulus? What is the nature of the attachment of the protein kinase to the membrane? Is the protein kinase specifically associated with other proteins? What is the identity of the protein substrates?

Continuing research will hopefully resolve some of the above points.

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BOUND PUBLICATIONS

TYROSINE SPECIFIC PROTEIN KINASES IN PLANT TISSUES

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Until 1979 protein kinases were considered to use only serine and threonine as substrates, although some work had been carried out on the so called 'acid labile' histone phosphates thought to be phosphohistidine and phospholysine (Chen et al. 1974). The discovery of an ability to phosphorylate tyrosine in Polyoma tumor antigen immunoprecipitates (Eckhart et al. 1979) opened a door to the understanding of cell growth control. Numerous other tyrosine specific protein kinases have now been discovered in animals, these include the receptors of insulin, epidermal growth factor, platelet derived growth factor (Krebs 1983), 17-β-estradiol (Migliaccio et al. 1984) and numerous retroviral oncogene products. To date no tyrosine kinase activity has been reported for any plant material.

In some cases the overall level of phosphotyrosine in animal cells may be as low as 0.05% of the total phosphoamino acid content (Sefton et al. 1982). Clearly, techniques used to reveal its presence must either separate or exclude phosphoserine and phosphothreonine. Three basic methods have been employed to identify in vitro tyrosine kinase activity in plant material using [γ-^{32}P]ATP. Each approach will now be discussed in turn.

Differential Stability of Phosphoserine, Phosphothreonine and Phosphotyrosine in Hot Alkali

This method relies upon the higher stability of the phosphodiester bond to tyrosine as compared to serine and threonine in the presence of hot alkali (Plimmer 1941). However, the original data applies to the free phosphoamino acids and others have found that phosphothreonine also shows a degree of stability when present in certain proteins (Cooper and Hunter 1981). Thus, the persistence of labelled bands in alkali treated polyacrylamide gels, revealed by autoradiography, is not an entirely reliable means of assessing the presence of phosphotyrosine unless each resistant band is subjected to hydrolysis and phosphoamino acid analysis.

Hydrolysis of Labelled Total Protein and Two Dimensional Phosphoamino Acid Analysis

Both crude membrane and ammonium sulphate precipitated fractions were prepared from plant material and after labelling were subjected to hydrolysis in 6N HCl and two dimensional separation by thin layer electrophoresis. Autoradiography was used to locate spots comigrating with phosphoamino acid markers.

Experiments of this type have been carried out using pea bud, pea root, zucchini hypocotyl and crown gall callus as the starting material. Whilst large amounts of phosphoserine and in some cases smaller amounts of phosphothreonine were observed in none of these experiments was any phosphotyrosine detected.

Artificial Substrates as a Means to Amplify Tyrosine Kinase Activity

Low levels of phosphotyrosine could be the result of the presence of few sites suitable for phosphorylation. The use of artificial substrates should supply the enzyme(s) with unlimited, but not necessarily ideal, sites. Such substrates must be easily isolated/assayed and if peptides must not contain serine or threonine. Angiotensin II and tyramine are suitable and readily available substrates. Free tyrosine is not soluble in water to any great extent. The phosphorylated products are isolated after trichloroacetic acid (TCA) precipitation of protein and hydrolysis of remaining [-^{32}P]ATP (Braun et al. 1983).

Both angiotensin II and tyramine were found to reduce the amount of label isolated with TCA soluble material for pea membranes and soluble fraction proteins. However, this cannot be taken as evidence that tyrosine kinase activity is absent since products must also be identified. Phosphorylated angiotensin II could not be identified in high voltage paper electrophoretic separations of the products and similarly, two dimensional thin layer electrophoretic separations revealed no phosphotyramine.

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CALCIUM/CALMODULIN DEPENDENT MEMBRANE BOUND PROTEIN KINASE

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Introduction

In recent years it has become apparent that the calcium ion plays a key regulatory role in plant cell metabolism. The details of the molecular events which contribute to this function are beginning to emerge. In animals it has been convincingly demonstrated that a common response to a variety of cell surface stimuli is a transient increase in cytoplasmic calcium concentration (Rasmussen & Barrett 1984). These alterations in cytoplasmic calcium concentration are interpreted by a number of calcium binding proteins. Of these the best characterized is calmodulin which has also been shown to participate in a variety of physiological processes in higher plants (Dieter, 1984). Other calcium binding proteins include enzymes whose activity is modulated by physiological concentrations of calcium. Among these enzymes, the protein kinases because of their known regulatory function are potentially of great importance in plant cells.

Since the first reports of membrane bound and soluble calcium activated protein kinases in plants (Hetherington & Trewavas 1982) (Polya & Davies 1982), these enzymes have been found in a number of species, tissues and are widely distributed within the cell. (Polya,Davies & Micucci 1983) (Salimath & Marme 1983) (Hetherington & Trewavas 1984) (Polya & Micucci 1984) (Blowers, Hetherington & Trewavas 1985). Calcium dependent protein kinases have been found in both soluble and particulate fractions, however with one exception the in vivo substrates for these enzymes are not known. The enzyme quinate : NAD^+ oxidoreductase has been shown to be regulated by phosphorylation/dephosphorylation catalysed by a calcium dependent protein kinase (Ranjeva et al 1983).

In this paper we will be restricting ourselves to discussion of membrane bound calcium dependent protein kinases. The role of membrane bound protein kinases in plants is not well understood. However an important role for these enzymes may well be anticipated from two recent sets of findings. Firstly it has been reported that auxins, cytokinins and gibberellins (Elliot et al 1983) and abscisic acid (de Silva, Hetherington and Mansfield 1985) require calcium for their activity and secondly that exogenous application of auxin influences the pattern of protein phosphorylation in soybean membranes (Morre, Morre and Varnold 1984).

The remainder of this paper will consider the properties of a protein kinase from pea shoot membranes.

Materials and Methods

Peas (Pisum sativum L.cv. Feltham First) were grown in the dark at 20-22°C for 12 days. The experimental material was the bud which contains the unexpanded 4th and 5th leaves.

Membrane preparation and phase partitioning : membranes were prepared as previously described (Hetherington, Trewavas 1984). An acetone solubilized membrane fraction was prepared from these membranes as described in (Blowers, Hetherington & Trewavas 1985). A plasma membrane enriched fraction was prepared by the procedure of Yoshida et al (1983). Total membrane was partitioned in 4g phases containing 5.6% (w/w) each of polyethylene glycol (Av. Mr.3,350) and dextran (Av.Mr. 472,000). Phases were thoroughly mixed and left to settle. The diluted upper and lower phases were centrifuged at 150000 x g for 1h.30 min and the resulting resuspended upper phase pellet referred to as the plasma membrane enriched fraction and the lower phase as the residual membrane fraction.

Electrophoresis, electroelution and blotting of proteins: PAGE of labelled and unlabelled proteins in denaturing and non-denaturing conditions and electroelution of proteins was carried out as described in Hetherington & Trewavas (1982) and Blowers, Hetherington & Trewavas (1985). Proteins separated using non-denaturing PAGE were transferred to nitrocellulose membranes (pore size 0.2 µm) using a Bio-Rad Transblot cell overnight (30V, 0.1A @ 4°C) with a 25mmol.dm^{-3} Tris, pH 8.3, 192mmol dm^{-3} glycine buffer system.

Detection of protein kinase activity on nitrocellulose membranes : After gel blotting nitrocellulose membranes were incubated for 30 mins in 0.5% (w/v) BSA, 0.9% (w/v) NaCl, 10mmol dm^{-3} Tris pH 7.5. The saturated membranes were washed 3 times in the above solutions without BSA. The membranes were then incubated in plastic bags containing appropriate buffer and approx 370 KBq γ-[^{32}P] ATP under the conditions indicated. On completion the membranes were washed overnight in 10% TCA, 20mmol dm^{-3} sodium pyrophosphate, 10mmol dm^{-3} EDTA. After boiling for 15 mins in fresh TCA mix and 30 min cooling the membranes were washed and filtered 2 times with TCA mix and air dried. Labelled proteins were detected by autoradiography.

Protein kinase assays, phosphoamino acid analysis and protein determinations: For membrane preparations protein kinase was assayed using the cellulose disc assay previously described (Hetherington and Trewavas 1982) using γ-[^{32}P]-ATP. For the purified enzyme, assays were performed exactly as described by Roskoski (1983) on Whatman P81 phosphocellulose strips (1x2cm). Incorporated radioactive phosphate was determined by Cerenkov counting of each disc or strip in 5 ml of distilled water. Phosphoamino acid analysis and protein determinations were carried out as detailed in Blowers, Hetherington & Trewavas (1985).

Results and discussion

We originally detected a membrane bound protein kinase in pea buds which was activated by micro molar concentrations of free calcium. Our subsequent studies revealed that the kinase activity was present throughout the 12 day old pea plant. However since the greatest calcium activation was associated with bud tissue (up to a maximum of 20 fold but typically some 6-7 fold) we chose this tissue as our experimental material.

We considered that the characterization and purification of the calcium activated membrane bound protein kinase (Ca^{2+}PK) would be significant steps towards understanding its physiological function. Specifically we attempted to answer two questions, first where at the subcellular level was the Ca^{2+}PK located and secondly was it regulatd by calmodulin? We attempted to localize the Ca^{2+}PK activity using a number of techniques. Isopynic centrifugation revealed the presence of at least 2 components, one was a shoulder and had a density of 1.16g cm^{-3} the other

major component had a peak density of 1.136g cm^{-3}. This latter density is characteristic of pea epicotyl plasma membrane. We confirmed these results using the technique of Rasi-Caldogno et al (1982) devised for pea tissue. These results indicated that protein kinase activity was associated with a number of subcellular fractions, however best calcium activations were observed in plasma membrane enriched fractions (Hetherington & Trewavas 1984).

Recently, to extend our localization stu'ies we have made use of the technique of phase partitioning. Figure 1 sh that the upper plasma membrane enriched phase contains much higher 6 fold) CaPK activity. Importantly the upper phase contains only one tenth of the total protein subjected to phase partitioning.

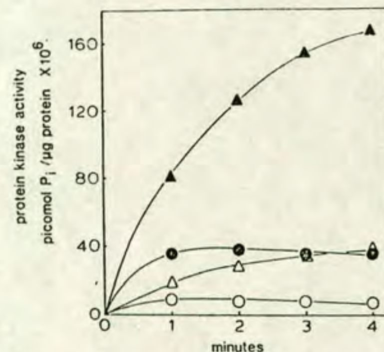

Fig.1. Phosphorylation of proteins in membrane fractions prepared by phase partioning. A membrane fraction was prepared from pea buds and separated by phase partitioning into an upper phase (plasma-membrane enriched) and a lower phase residual membrane fraction. Closed symbols incubation contains 100µmol dm^{-3} $CaCl_2$, open symbols no $CaCl_2$ present, triangles show upper plasma membrane enriched phase, circles show lower residual membrane phase.

We have also investigated possible calmodulin (CaM) dependence of the Ca^{2+}PK. Initially we were only able to demonstrate inhibition of Ca^{2+}PK activity by high concentrations of Trifluoperazine (TFP) and stimulation by large amounts of CaM (Hetherington & Trewavas 1982). We interpreted this difficulty as being due to contamination of the membrane preparations by endogenous CaM, although we subsequently managed to improve the TFP inhibition by preincubation of the membrane preparations in 2mmol dm^{-3} EGTA.

In order to further characterize the enzyme we attempted solubilization using a variety of agents (Na-deoxycholate, NP40, CHAPS). However the best method for both solubilizing the Ca^{2+}PK while retaining its calcium dependence was the acetone procedure devised by Venis (1977) as illustrated in Fig.2.

Interestingly acetone solubilized material was more sensitive to TFP but showed no response to added calmodulin.

We had previously obtained evidence that pea bud membranes contained more than one kinase. Did our solubilized preparation contain more than

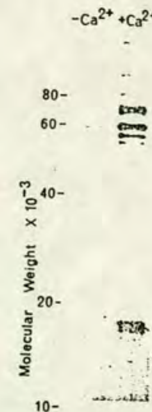

Fig.2. Autoradiograph of proteins phosphorylated by acetone solubilized protein kinase incubated in the absence and presence of calcium ions separated by SDS PAGE.

one kinase? We utilized the procedures of non-denaturing PAGE coupled with Western blotting to answer this question. Fig.3 shows the results of a non denatured sample of labelled protein separated by non denaturing PAGE.

To detect protein kinase activity we took advantage of the observations that protein kinases are known to autophosphorylate and also they frequently co-purify with their endogenous substrates. After Western transfer of non-denatured proteins to nitrocellulose, enzyme activity was retained. A single phosphorylated band appears on the filter indicating the presence of protein kinase. We were subsequently able to demonstrate by phospho-amino acid analysis that the labelled band resulted from kinase activity and not from non specific binding of ^{32}P to protein. Approximately 90% of the phosphorylation was on serine residues with the remainder on either threonine or tyrosine (Blowers, Hetherington & Trewavas 1985).

By running non-denaturing gels of the acetone solubilized Ca^{2+}PK and including a single labelled lane we were able to locate the protein kinase band in other non labelled lanes. These bands were removed and the protein kinase electroeluted. The isolated protein was tested for Ca^{2+}/calmodulin dependence in the absence of exogenous substrate. Fig 4 indicates that the enzyme shows a 3 fold activation in the presence of calcium and calmodulin. Importantly little activity is seen in the presence of calcium alone.

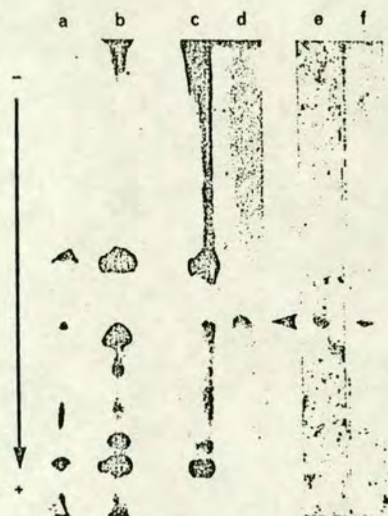

Fig.3. Separation of labelled peptides in acetone solubilized protein kinase preparations by non denaturing gel electrophoresis and Western blotting of protein kinase activity.

Tracks a and b: acetone solubilized membrane proteins were phosphorylated in the absence (track a) or presence (track b) of 100 umol.dm^{-3} $CaCl_2$ and were loaded directly on a non-denaturing gel separated for 16h at 4°C and subsequently autoradiographed. Track c : proteins labelled as above and blotted to nitrocellulose. Tracks d-f solubilized proteins were separated as above unlabelled. The gel containing tracks c-f was blotted onto nitrocellulose and active protein kinase detected by incubating the nitrocellulose in γ-[^{32}P]-ATP. In tracks d and e the nitrocellulose was additionally incubated in 100 umol dm^{-3} $CaCl_2$ and track f in the absence of $CaCl_2$.

When the kinase containing band was electroeluted from non-denaturing gels, incubated with γ-[^{32}P]-ATP and separated on SDS gels a number of polypeptide bands were visible after coomassie blue or silver staining. However, autoradiography revealed the presence of only one phosphorylated band, and this did not correspond with any of the protein stainable bands. We do not know whether these components represent subunits of the native protein kinase or polypeptides which co-purify with the enzyme. Thus the phosphorylated component may be the result of autophosphorylation or may represent the co-purified endogenous substrate. However, it is clear that the latter component is present in low amounts (Fig.5).

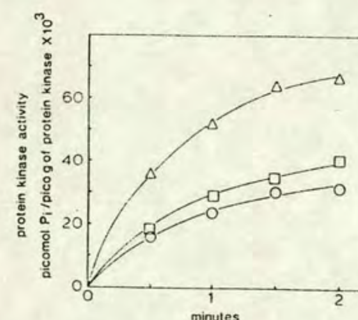

Fig.4. Activation of purified membrane bound protein kinase activity by calmodulin: acetone solubilized membrane proteins were separated by non denaturing gel electrophoresis, the band containing the protein kinase was located, excised and the active enzyme electroeluted. This was then incubated in γ-[^{32}P]- ATP in the absence of Ca^{2+} (o) or with 100 mol dm^{-3} $CaCl_2$ (□) or with 100 mol dm^{-3} $CaCl_2$ 100 g ml^{-1} bovine calmodulin (△).

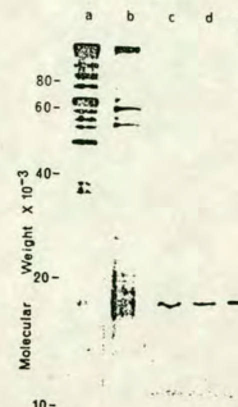

Fig.5. SDS PAGE of phosphorylated polypeptides from various protein kinase preparations: Pea bud membranes were prepared and separated by phase partitioning into a lower residual membrane preparation and an upper membrane enriched fraction. Phosphorylation with γ-[^{32}P] ATP was carried out in the presence of 100 μmol dm^{-3} free Ca^{2+} and the labelled proteins separated by SDS gel electrophoresis. Track a, autoradiograph of the residual membrane protein phosphorylation and track b the plasma membrane enriched fraction.

Acetone solubilized protein kinase was separated by non-denaturing gel electrophoresis, the active bands excised electroeluted and allowed to autophosphorylate with γ-[^{32}P] ATP. Track c no $CaCl_2$, track d 100 μmol dm^{-3} free Ca^{2+}, track e 100 μmol dm^{-3} free Ca^{2+}, 50 $\mu g\ ml^{-1}$ bovine calmodulin. Notice the single band in c-e which is present only in the plasma membrane enriched fraction.

To summarise our results we have extensively purified a calcium calmodulin dependent protein kinase from pea bud membranes. All our evidence indicates that the enzyme is located in a plasma membrane enriched fraction. We feel that our previous failures to satisfactorily demonstrate calmodulin dependence were due to difficulty in depleting endogenous calmodulin from our membrane preparations.

Our future research will be concerned with investigating the function of the enzyme. Towards this goal we are currently raising antibodies to the purified enzyme.

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Isolation of plasma-membrane-bound calcium/calmodulin-regulated protein kinase from pea using Western blotting

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Abstract. Membranes isolated from pea buds contain protein-kinase activity which is greatly activated by low concentrations of calcium ions. This paper describes a simple purification of this enzyme with a novel means of detecting enzyme activity by Western blotting. The purified enzyme appears to autophosphorylate primarily on serine residues, is activated by bovine calmodulin and additional evidence from phase partitioning indicate most of this enzyme to be located in the plasma membrane.

Key words: Calcium – Calmodulin – *Pisum* (protein kinase) – Plasma membrane – Protein kinase.

Introduction

The importance of Ca^{2+}/calmodulin-regulated processes in plants is becoming increasingly apparent. Enzymes such as NAD kinase (Anderson et al. 1980) and quinate:NAD oxidoreductase (Ranjeva et al. 1983) show enhanced activity in the presence of Ca^{2+} and calmodulin. A number of plant protein kinases also show increased activity in the presence of Ca^{2+} and calmodulin (Hetherington and Trewavas 1982; Polya and Davies 1982; Refeno et al. 1982; Salimath and Marme 1983; Polya and Micucci 1984). The identity of the substrate(s) for these protein kinases has remained absent in all except one case; the protein kinase of Refeno et al. (1982) is known to phosphorylate and regulate the activity of quinate:NAD oxidoreductase.

It was considered that the identification, purification and characterization of a membrane-bound protein kinase would be an important step towards understanding its possible role in cellular signal transduction. We have previously reported the presence of calcium-dependent protein-kinase activity in membranes isolated from pea buds (Hetherington and Trewavas 1982). This activity was, with difficulty, reduced by calmodulin antagonists and appeared to have a major location in a light-membrane preparation enriched in plasma membrane (Hetherington and Trewavas 1984).

In this report we present the results of a partial purification of this membrane-bound, calcium-activated protein kinase from pea buds. The enzyme preparation appears to autophosphorylate in a calcium/calmodulin-activated fashion and further evidence is presented to suggest a plasma-membrane location. These results are in agreement with the idea that this enzyme is a likely early interpreter of an influx of extracellular calcium.

Abbreviations: PAGE = polyacrylamide-gel electrophoresis; SDS = sodium dodecyl sulphate

Material and methods

Plant material. Peas (*Pisum sativum* L. cv. Feltham First) were grown in moist vermiculite in the dark at 20–22° C for 12 d. The experimental material was the bud which contains the unexpanded fourth and fifth leaves.

Chemicals. [γ-^{32}P]ATP (specific activity 111 TBq·mmol^{-1}) was obtained from Amersham International plc (Amersham, Bucks., UK). Other chemicals were obtained from Sigma London Chemical Co. (Poole, Dorset, UK). Bovine calmodulin was obtained from Calbiochem (Bishops Stortford, UK).

Membrane preparation and acetone precipitation. Washed cell membranes were prepared and protein kinase solubilised as previously described (Hetherington and Trewavas 1984). Acetone precipitates were prepared from these membranes using a modification of the method of Venis (1977). After resuspension in buffer [300 mmol·dm^{-3} Sorbitol, 6 mmol·dm^{-3} 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), 6 mmol·dm^{-3} 2-[N-morpholino]ethanesulphonic acid (Mes), pH 7.2 and 0.2 mmol·dm^{-3} disodium ethylenediaminetetraacetic acid (Na_2EDTA)], the membranes were added dropwise to twenty volumes of ice-cold acetone and left to stand for 30 min. A pellet was produced by centrifugation for 5 min at 3000 *g* and resuspended in the same buffer plus 20% glycerol. After thorough homogenisation and standing on ice for 15 min the resuspended pellet was then centrifuged at 40000 *g* for 30 min. The pellet was discarded. The final supernatant is referred to as the acetone-solubilised membrane fraction.

Phase partitioning of membranes. The methods used were those of Yoshida et al. (1983). Total membrane was partitioned in 4-g phases containing 5.6% (w/w) each of polyethylene glycol (approx. M_r 3350) and dextran (average M_r 472000). Phases were thoroughly mixed by 30 inversions and left to settle for 10 min at 0° C. Brief centrifugation in a bench centrifuge was used to aid phase separation. The diluted upper and lower phases were centrifuged at 150000 *g* for 1.5 h, and the resulting resuspended upper-phase pellet is referred to as the plasma-membrane-enriched fraction and the lower phase as the residual-membrane fraction.

Gel electrophoresis. Sodium-dodecyl-sulphate (SDS) polyacrylamide-gel electrophoresis (PAGE) of labelled proteins was carried out using 12% gels as previously described, and labelled bands detected by autoradiography (Hetherington and Trewavas 1982). Polyacrylamide-gel electrophoresis under non-denaturing conditions was carried out using 7% gels (20·16·0.1 cm^3) and a discontinuous gel buffer system of 375 mmol·dm^{-3} Tris-sulphate pH 8.3 and an electrode buffer 25 mmol·dm^{-3} Tris/192 mmol·dm^{-3} glycine, and separations were carried out at 4° C for 16 h at 7.5 V·cm^{-1}. For analysis of labelled proteins under non-denaturing conditions, the membrane preparations were labelled in the presence of 10% (w/v) glycerol, added to the sample wells of the polyacrylamide gel and the voltage applied. When necessary, i.e. before electroelution, gels were stored at 4° C overnight. Labelled proteins were detected by autoradiography as before. Silver staining was performed according to Goldman et al. (1980).

Electroelution of protein from non-denaturing gels. Protein-kinase-containing bands were cut from the polyacrylamide gels and homogenised in a small volume of the electrode buffer (25 mmol·dm^{-3} Tris-sulphate, 20% glycerol, pH 8.0). The homogenate was contained in a modified tube gel apparatus with nylon mesh and protein eluted overnight at 4° C into dialysis tubing with a constant 150 V. At the end of the elution period the polarity was reversed for 5 min to reduce the loss of protein caused by its tendency to stick to the dialysis tubing (Tijssen and Kurstak 1979).

Protein blotting. Proteins separated using non-denaturing PAGE were transferred to nitrocellulose membranes (pore size 0.2 µm, Schleicher and Schull, Dassel, FRG) using a Bio-Rad Transblot cell (Bio-Rad Laboratories, Richmond, Cal., USA). Transfer took place overnight (30 V, 0.1 A at 4° C) using a buffer system of 25 mmol·dm^{-3} Tris pH 8.3, 192 mmol·dm^{-3} glycine.

Detection of protein-kinase activity on nitrocellulose membranes. After gel blotting, nitrocellulose membranes were incubated for 30 min in 0.5% (w/v) bovine serum albumin (BSA) 0.9% (w/V) NaCl, 10 mmol·dm^{-3} Tris pH 7.5. The saturated membranes were washed three times in the above solution without BSA. The membranes were then incubated in plastic bags containing appropriate buffer and approx. 370 kBq [γ-^{32}P]ATP under the conditions indicated.

On completion, the membranes were washed overnight in 10% trichloroacetic acid (TCA), 20 mmol·dm^{-3} sodium pyrophosphate, 10 mmol dm^{-3} EDTA. After boiling for 15 min in fresh TCA mix and 30 min cooling, the membranes were washed a further two times with TCA mix and air-dried. Labelled proteins were detected by autoradiography.

Phosphoamino-acid analysis. The area of the nitrocellulose containing labelled protein-kinase activity was excised and hydrolyzed in the presence of 6 mol·dm^{-3} HCl for 24 h at 100° C. The resuspended hydrolyzate was then separated on Whatman (Springfield Mill, Maidstone, Kent, UK) 3 MM paper by electrophoresis in pH 1.9 buffer (ethanoic acid:methanoic acid:H_2O::78:25:897).

Protein determination. The method of Bearden (1978) was employed with absorbance measurement at a single wavelength (595 nm) and BSA as standard.

Protein-kinase assays. For membrane preparations the cellulose-disc assay previously described was employed (Hetherington and Trewavas 1984) using [γ-^{32}P]ATP. For the purified enzyme, assays were performed exactly as described by Roskoski (1983) on Whatmann P81 phosphocellulose strips (1·2 cm^2). Incorporated radioactive phosphate was determined by Cerenkov counting of each disc or strip in 5 ml of distilled water. Free calcium was estimated as indicated by Hetherington and Trewavas (1982).

Results

Calcium dependence of solubilised membrane-located protein kinase. In a previous paper, we described and characterised a membrane-bound protein kinase from pea which was activated by low concentrations of calcium ions (Hetherington and Trewavas 1984). In addition, we reported the solubilization of approx. 30% of the total protein-kinase activity using acetone, although with reduced calcium dependence. Figure 1 shows the autoradiograph of an SDS-PAGE separation of acetone-solubilized proteins labelled in the absence or presence of calcium ions. Labelled proteins were detected by autoradiography of the dried-down gel. In the presence of calcium, several bands covering a range of molecular weights are labelled, note particularly the group of bands around 18 kDa. In the absence of calcium, the labelling is less intense and restricted mainly to a single band with a molecular weight of approximately 60 kDa. Other bands can be detected with prolonged exposure. The preparation and type of bands labelled is very different from whole membranes and shown by us previously, but Fig. 1 shows that the solubilised protein kinase, importantly, retains a selectivity in the proteins it phosphorylates. At this stage the protein kinase shows little or no response to added calmodulin.

Further purification by non-denaturing gel electrophoresis and detection of separated protein-kinase activity by Western blotting. In our previous paper,

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Fig. 1. Autoradiography of proteins labelled by acetone-solubilised protein kinase incubated in the presence and absence of calcium ions and separated by SDS-PAGE. Pea-bud membranes were prepared, and precipitated with acetone. After suspension and removal of insoluble material, the solubilised protein kinase was incubated in [γ-^{32}P]ATP in the presence and absence of 100 μmol·dm^{-3} fee Ca^{2+} for 1 min. After addition of an equal volume of sample buffer (containing 4.7% SDS, 10% 2-mercaptoethanol, 20% Ficoll (M_r approx. $4\cdot10^5$) and 125 mmol·dm^{-3} Tris, pH 6.8) the sample was boiled for 10 min before gel separation and autoradiography

we indicated that pea-bud membranes may contain more than one protein kinase. Did our solubilised preparation contain more than one enzyme? We have used non-denaturing gel electrophoresis to try and resolve this issue. As a means of detecting active protein kinase on the separated gels, the proteins were Western blotted and attempts made to see if enzyme activity was retained after such a procedure. In addition, we took advantage of the fact that many protein kinases autophosphorylate to use labelling with [γ-^{32}P]ATP as a future and

easy means of locating the separated enzyme on non-denatured gels.

Figure 2 summarises some twelve successful experiments in this respect. Fig. 2 (tracks a and b) shows the result of PAGE separation of acetone-solubilized proteins performed under non-denaturing conditions on a 7% gel. Proteins were labelled with [γ-^{32}P]ATP in the absence (track a) or presence (track b) of calcium ions, loaded directly, separated and detected by autoradiography of the dried-down gel. At least four major labelled bands are present in track b and one of these is novel to the calcium incubation itself, although there is a general increase in phosphorylation of all of them.

To detect possible protein-kinase activity, both labelled and unlabelled proteins separated on other non-denaturing gels were transferred to nitrocellulose membranes by Western blotting in a Bio-Rad Transblot cell. The subsequent blotted nitrocellulose membranes were site-saturated with bovine serum albumin and incubated with [γ-^{32}P]ATP in the absence or presence of calcium ions. After extensive hot-acid washing and drying the membranes were autoradiographed. Labelled bands should reveal the location of protein-kinase activity.

Figure 2 (tracks c–f) shows the results of such investigations. Lanes c and d represent adjacent tracks of a single gel. Acetone-solubilized proteins were run on a non-denaturing 7% polyacrylamide gel, but prior to running, track c was labelled with [γ-^{32}P]ATP in the presence of calcium ions. Track d was unlabelled. These lanes were transferred to nitrocellulose, and the nitrocellulose membrane was then sealed in a plastic bag in the presence of [γ-^{32}P]ATP and 100 μmol·dm^{-3} free calcium and incubated at room temperature for 2 h. Upon removal from the bag and after washing, the nitrocellulose was subjected to autoradiography. Track c shows the normal phosphorylated bands transferred to nitrocellulose, track d shows that one of these is a protein kinase which has retained activity after blotting. In tracks e and f the unlabelled, but nitrocellulose blotted, lanes were incubated under identical conditions except for the absence or presence of 100 μmol·dm^{-3} free calcium. The same band is observed in each case and no calcium activation is now apparent. So far as we are aware this is the first report of retention of enzyme activity after such a procedure.

To test whether the labelled band in tracks d–f was the result of protein-kinase activity or whether it resulted from some non-specific binding of [γ-^{32}P]ATP, the band was cut out of the nitrocellu-

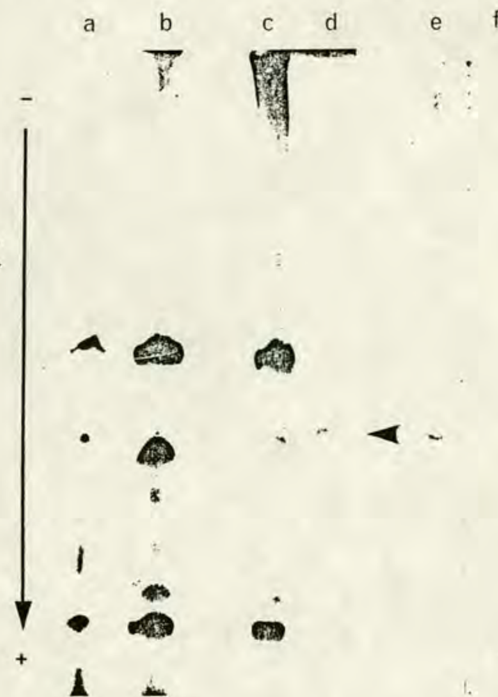

Fig. 2. Separation of labelled peptides in acetone-solubilised protein-kinase preparations by non-denaturing gel electrophoresis and Western blotting of protein-kinase activity. *Tracks a, b:* pea-bud membranes were isolated and an acetone-solubilised fraction prepared. Proteins were phosphorylated by incubating in [γ-^{32}P]ATP and in the absence (*track a*) and presence (*track b*) of 100 μmol·dm^{-3} free Ca^{2+} for 1 min. Samples were loaded directly on a non-denaturing gel ($20\cdot16\cdot0.1$ cm^3) and separated for 16 h at 4° C. The gel was dried down and autoradiographed. *Track c:* proteins labelled and separated as above and blotted to nitrocellulose. *Tracks d–f.* solubilised proteins were separated as above but unlabelled. The gel containing tracks c–f was blotted onto nitrocellulose (as in Material and methods) and active protein kinase detected by incubating the nitrocellulose in [γ-^{32}P]ATP for 2 h at room temperature. In *tracks d* and *e* the nitrocellulose was additionally incubated in 100 μmol·dm^{-3} free Ca^{2+} and *track f* in the absence of $CaCl_2$

lose membrane and hydrolyzed in the presence of 6 mol·dm^{-3} HCl at 100° C for approx. 24 h. Figure 3 shows a high-voltage electrophoretic separation of the hydrolysate. It can be seen that ^{32}P is incorporated into phospho-amino acids and is therefore the result of protein-kinase activity. Approximately 90% of the phosphorylation was on serine residues with the remainder on either threonine or tyrosine.

Calmodulin dependence of purified protein kinase. By running non-denaturing gels of the acetone-solubilised protein kinase and including a single labelled lane, it was now found possible to locate the protein-kinase-containing band in other non-labelled lanes. Such bands were cut out, bulked and the protein electroeluted into a dialysis bag using a modified tube gel apparatus. The eluate thus represented a purification of the protein kinase.

The isolated enzyme was tested for calcium- and calmodulin-dependent activity in the absence of exogenous substrate. Figure 4 presents the results of phosphocellulose strip assays (Roskoski 1983) performed in the absence or presence of 100 μmol·dm^{-3} free calcium or in the presence of calcium with bovine calmodulin at 50 μg·ml^{-1}. Duplicate assays were performed and suitable blanks substracted.

The protein kinase shows a three fold activation (taken from initial rates) in the presence of calcium and calmodulin and does not phosphorylate the added calmodulin (see Fig. 6, track c). Little activation is seen in the presence of calcium alone, confirming the data of Fig. 2e, f and contrasting with the initial acetone-solubilised protein-kinase preparation.

Possible location of purified calcium-dependent protein kinase in the plasma membrane, and further purification. Our previous examination of the membrane location of calcium-regulated protein kinase has indicated a major locale in plasma-membrane-enriched fractions. Figure 5 examines

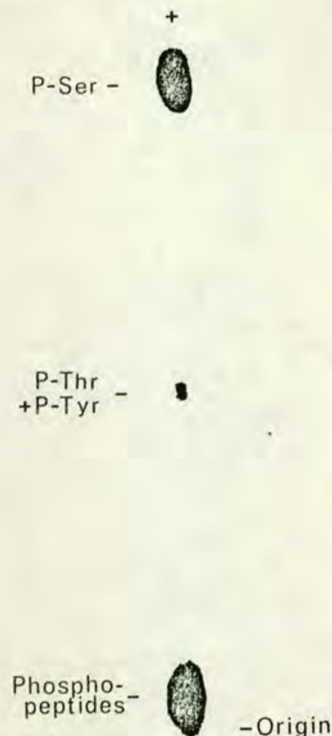

Fig. 3. Autoradiograph of amino acids labelled by the nitrocellulose-bound purified protein-kinase activity. Acetone-solubilised protein kinase was separated by non-denaturing gel electrophoresis and Western blotted to nitrocellulose. After incubation in [γ-^{32}P]ATP and washing, the one labelled band (equivalent to that shown in Fig. 2d–f) was excised and hydrolyzed in 6 mol·dm^{-3} HCl at 100° C for 24 h. The resulting hydrolyzate was separated by high-voltage paper electrophoresis at pH 1.9. The mobility position of unlabelled markers phosphoserine, phosphothreonine and phosphotyrosine are indicated at the side

the distribution of calcium-regulated protein kinase in membranes separated by phase partitioning (Albertson 1958). This technique which reportedly gives a higher enrichment of plasma membrane than other methods has been shown to work with numerous tissues. Figure 5 shows the kinetics of

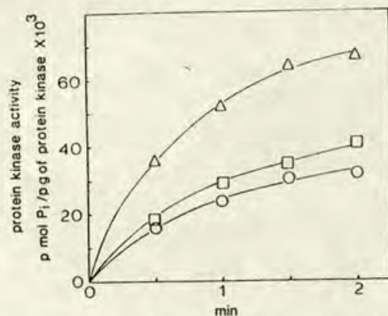

Fig. 4. Activation of purified membrane-bound protein kinase activity by calmodulin. Acetone-solubilised membrane proteins were separated by non-denaturing gel electrophoresis; the band containing the protein kinase was located, excised and the active enzyme electroeluted. This was then incubated in [γ-^{32}P]ATP in the absence of calcium (O) or with 100 µmol·dm^{-3} free Ca^{2+} (□) or with 100 µmol·dm^{-3} free Ca^{2+}, 50 µg·ml^{-1} bovine calmodulin (△)

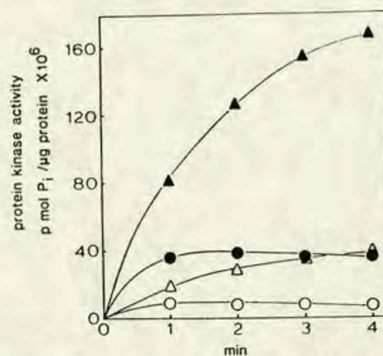

Fig. 5. Phosphorylation of membrane-bound proteins in membrane fractions prepared by phase partitioning. A membrane fraction was prepared from pea buds and separated by phase partitioning into an upper phase (plasma-membrane-enriched) and a lower phase (residual membrane) fraction. Phosphorylation was conducted in both membrane preparations using [γ-^{32}P]ATP at 0° C. *Closed symbols*, incubation contains 100 µmol·dm^{-3} free Ca^{2+}; *open symbols*, no $CaCl_2$ present; △, upper, plasma-membrane-enriched phase; ○, lower, residual-membrane phase

labelling with [γ-^{32}P]ATP of membranes divided into plasma-membrane-enriched and residual-membrane fractions as described in Material and methods. There are marked differences in the kinetics of labelling. On a specific-activity basis, protein-kinase activity is much higher (about six fold)

and continues for a much longer period in the plasma-membrane-enriched fraction. Calcium dependence of protein-kinase activity is evident in both fractions. It should be noted that the upper phase contains only approximately one tenth of the total protein subjected to phase partitioning.

The data in Fig. 6 indicate that our solubilised protein kinase is located in the plasma-membrane-enriched fraction. Tracks a and b show the SDS-gel separations of the proteins labelled in the presence of calcium ions in both the 'residual' and plasma-membrane-enriched phases and detected by autoradiography.

At least two major labelled bands (approx. M_r 65 and 50 kDa) of the 'residual' membranes (track a) are represented to a lesser extent in the plasma-membrane-enriched phase (track b). Conversely and importantly, several bands of lower molecular weight (approx. 17, 18 and 19 kDa) are apparent to a much greater extent in the plasma-membrane-enriched phase. The total membrane preparation, prior to phase partitioning, is essentially identical to the residual phase both in terms of kinetics and the proteins labelled.

Tracks c, d and e show the labelled products produced when the calcium-regulated protein kinase, electroeluted from a non-denaturing gel is incubated in [γ-^{32}P]ATP under three different conditions. Track c is incubated in the absence of Ca^{2+}, track d in the presence of Ca^{2+} and track e in the presence of calcium and calmodulin.

In every case a single major band is seen to be labelled (M_r approx. 18 kDa), and the calmodulin acitvation (track e) is readily apparent. A minor band of approx. 19 kDa may also be observed with longer exposure of the autoradiograph. The autophosphorylated protein kinase band appears to align with a band seen to be enhanced only in the plasma-membrane-enriched fraction again indicating its major location in this cell membrane. Coomassie staining of such SDS gels revealed that the protein kinase does not phosphorylate the added calmodulin.

With the above knowledge, a combination of phase partitioning and acetone solubilisation was used to obtain further purification of the 'crude' sample. Figure 7 presents the results of combining these two procedures. All lanes in Fig. 7 are derived from PAGE under non-denaturing conditions on a 7% polyacrylamide gel of the phase partitioned and solubilised fraction. Silver staining (track a) reveals a staining band in the vicinity of, but not coinciding with the protein kinase. Track b shows an autoradiograph of a similar labelled separtion. (The degree of purification obtained can

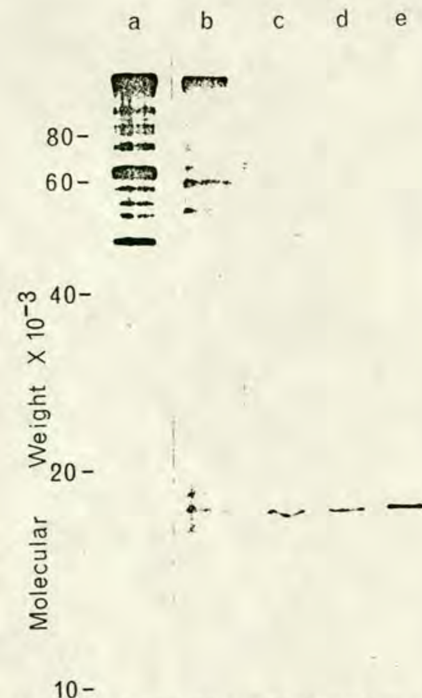

Fig. 6. Possible location of solubilised and purified membrane-bound protein kinase in plasma-membrane-enriched fraction. Pea-bud membranes were prepared and separated by phase partitioning into a lower, residual-membrane preparation and an upper, plasma-membrane-enriched fraction. Phosphorylation was carried out with [γ-^{32}P]ATP in the presence of 100 µmol·dm^{-3} free Ca^{2+} and the labelled proteins separated by SDS gel electrophoresis. *Track a* shows the autoradiograph of the residual-membrane protein phosphorylation and *track b* the plasma-membrane-enriched fraction. Acetone-solubilised protein kinase was separated by non-denaturing gel electrophoresis, the active bands were excised, electroeluted and allowed to autophosphorylate with [γ-^{32}P]ATP. *Track c*, no $CaCl_2$; *Track d*, 100 µmol·dm^{-3} free Ca^{2+}; *Track e*, 100 µmol·dm^{-3} free Ca^{2+}, 50 µg·ml^{-1} bovine calmodulin. The incubations were carried out for 1 min at 0° C before denaturing, and separation by SDS gel electrophoresis and autoradiography. Notice the single band in c–e which is present only in the plasma-membrane-enriched fraction

be seen by comparing Fig. 7, track b with Fig. 2, track b). The protein kinase, which autophosphorylates when blotted to nitrocellulose, track c, is found to coincide with a minor labelled band in track b. This band is seen to partition out of the total-membrane prepartion whilst the major la-

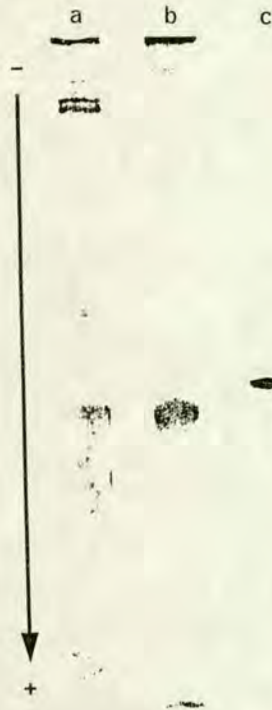

Fig. 7. Separation of labelled peptides in acetone-solubilised phase-partition preparations by non-denaturing gel electrophoresis and Western blotting of protein-kinase activity. *Track a*: pea bud membranes were isolated and an acetone-solubilised fraction prepared from the plasma-membrane-enriched phase-partition fraction. Samples were loaded directly onto a non-denaturing gel (20·16·0.1 cm^3) and separated for 16 h at 4° C. The gel was silver stained and dried down. *Track b*: proteins were isolated as above and phosphorylated by incubating in [γ-^{32}P]ATP in the presence of 100 $\mu mol \cdot dm^{-3}$ free Ca^{2+} for 1 min. Labelled proteins were separated as above and the dried-down gel autoradiographed. *Track c*: solubilised proteins were separated as above but unlabelled. After blotting to nitrocellulose the active protein kinase was detected by incubation in [γ-^{32}P]ATP in the presence of 100 $\mu mol \cdot dm^{-3}$ free Ca^{2+} for 45 min. at 4° C. The autophosphorylating protein kinase was revealed by autoradiography

belled band is novel to this solubilized plasma-membrane-enriched fraction. The apparent lack of silver staining of the protein-kinase band and another faster running labelled band in the same preparation probably reflects the small amounts

of protein present in these two positions but conversely indicates extensive purification.

Discussion

This partial purification of a protein kinase from pea-bud membranes has provided evidence that its activity is both calcium and calmodulin dependent (Fig. 4). However, the possibility exists that the calmodulin activation is non-specific and that other components may be necessary for full activity. The demonstration of calmodulin dependence in intact membranes has proved difficult in the past and this probably results from the very high amounts of calmodulin retained in these membranes (Hetherington and Trewavas 1984). The presence of endogenous calmodulin and its lack of easy removal make the addition of exogenous calmodulin ineffective in activation until the enzyme has been further purified, and this has now been demonstrated.

The apparent autophosphorylating activity of the isolated protein kinase is not an unusual property of membrane-associated protein kinases – and could have a regulatory role. Many animal-polypeptide hormone receptors such as those for epidermal growth factor (Ushiro and Cohen 1980), platelet-derived growth factor (Ek et al. 1982) and insulin (Kasuga et al. 1982) show enhanced autophosphorylation on tyrosine residues in response to peptide binding. The estradiol receptor not only shows such enhanced autophosphorylation, but is also calcium/calmodulin dependent (Migliaccio et al. 1984).

In our previous paper (Hetherington and Trewavas 1984) describing this enzyme, we reported that by two relatively well-characterised procedures in pea the majority of the calcium-regulated protein-kinase activity was to be found in the plasma-membrane-enriched fractions. This paper adds a third method which entirely supports our earlier conclusion; predominantly the enzyme is found in this cellular location. Whether it is all there will be very difficult to demonstrate but the fact that it may be located there at all indicates that its major function may be interpretation of extracellular signalling such as through phytochrome, rather than signals coming through the symplast.

The combination of phase partitioning and solubilization clearly provides a rapid and simple means of purification and will be used in the future to provide a suitable preparation for the production of antibodies.

So far as we are aware this is the first report of retention of any enzyme activity on nitrocellu-

lose. Although protein-kinase activity can be detected in gel slices, such methods are slow and laborious and lack the elegance of the simple method described here. Retention of protein-kinase activity indicates that other enzymes too may retain activity under these conditions, thus greatly simplifying detection of enzymes separated by one- or two-dimensional gel electrophoresis. The adoption of such methods could greatly speed up the identification of unknown protein bands and spots on gels, perhaps one of the most pressing problems in present-day plant molecular biology.

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