The Calmodulin Stimulated ATPase of *Zea mays* L.



Thesis presented by

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Abstract

THE CALMODULIN-STIMULATED ATPase of *Zea mays* L.

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Maize coleoptile microsomal vesicles showing calmodulin-stimulated ATPase activity were isolated from 4.5 day old dark grown seedlings. Calmodulin-stimulated ATPase activity was maximal (8 nmoles min^{-1} (mg protein)$^{-1}$) at 0.35 μM, inhibited by orthovanadate (I_{50}=20 μM), and specific for ATP.

Calmodulin affinity chromatography was used to purify this ATPase after solubilisation with Triton X-100. Calmodulin-stimulated ATPase activity was present in the purified fraction, maximal stimulation (340 nmoles min^{-1} (mg protein)$^{-1}$) occurring at 0.3 μM calmodulin. After reconstitution into asolectin liposomes, maximal calmodulin-stimulated ATPase activity (500 nmoles min^{-1} (mg protein)$^{-1}$) occurred at 0.025 μM. Affinity chromatography using buffers containing asolectin produced true basal activities; maximal calmodulin stimulation was at 0.01 μM (100 nmoles min^{-1} (mg protein)$^{-1}$). These results suggest that a calmodulin-stimulated ATPase was purified from the microsomal fraction.

Inclusion of protease inhibitors (PMSF, chymostatin) during purification and electrophoresis yielded a polypeptide of 140,000 M_r, similar to the M_r of erythrocyte calmodulin-stimulated, calcium-pumping ATPase (CSCPA). Polypeptides of M_r 91,000, 77-69,000, 51,000, and 40,000 were also present. A monospecific polyclonal antibody raised against erythrocyte CSCPA recognised the 140,000 M_r polypeptide from maize, giving strong evidence that maize cells may contain a polypeptide similar to erythrocyte CSCPA.

The reaction mechanism of the proposed maize CSCPA was investigated. After purification in the presence of PMSF phosphorylation was present at 140,000 M_r; this turned over rapidly, was sensitive to hydroxylamine, dependent on calcium, inhibited by lanthanum and stimulated by calmodulin. This was consistent with formation of an acyl-phosphate intermediate, indicating that maize CSCPA is a P-type ATPase, having a reaction mechanism similar to that of the erythrocyte CSCPA.

A monoclonal antibody (EA6) was raised to maize CSCPA purified without PMSF; this antibody recognised intact maize CSCPA and inhibited calmodulin-stimulated ATPase activity in microsomal fractions. This antibody also bound to other polypeptides present in microsomal and purified fractions, permitting tentative identification of proteolysis products.

'He also said, "This is what the Kingdom of God is like. A man scatters seed on the ground. Night and day, whether he sleeps or gets up, the seed sprouts and grows, though he does not know how. All by itself the soil produces corn - first the stalk, then the ear, then the full grain in the ear. As soon as the grain is ripe, he puts the sickle to it, because the harvest has come." '

Mark 4: 26-29

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To My Parents

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Abbreviations

ABA abscisic acid

ADP adenosine 5'-diphosphate

ATP adenosine 5'-triphosphate

ATPase adenosine 5'-triphosphatase

$\mathbf{A_{280}}$ absorbance at 280 nm

$\mathbf{A_{450}}$ absorbance at 450 nm

$\mathbf{A_{650}}$ absorbance at 650 nm

BSA bovine serum albumin

CaM calmodulin

Ca^{2+}-ATPase calcium-stimulated/calcium pumping ATPase

(Ca^{2+}+Mg^{2+})-ATPase calcium/magnesium-stimulated ATPase

CCCP carbonylcyanide-m-chlorophenyl hydrazone

cDNA complementary DNA

c.p.m. counts per minute

CSCPA calmodulin-stimulated, calcium-pumping ATPase

CTP cytidine 5'-triphosphate

cAMP adenosine 3',5'-cyclic monophosphate

cCMP cytidine 2',3'-cyclic monophosphate

cGMP guanosine 3',5'-cyclic monophosphate

DAG diacylglycerol

DCCD N,N'-dicyclohexylcarbodiimide

DES diethylstilbestrol

DIDS 4,4 - diisothiocyano-2,2'-stilbenedisulphonate

DMSO dimethylsulphoxide

DNA deoxyribonucleic acid

DTT dithiothreitol

EDTA ethylenediaminetetraacetic acid

EGTA (ethylene-dioxy) diethylenedinitrilotetraacetic acid

ELISA enzyme-linked immunosorbent assay

ER endoplasmic reticulum

FBS foetal bovine serum

FCCP carbonylcyanide-p-trifluoromethoxyphenyl hydrazone

G-protein GTP-binding protein

GTP guanosine triphosphate

[γ^{32}P]-ATP ATP labelled in γ-position with ^{32}P

HAZA azaserine-hypoxanthine media supplement

HEPES N-2-hydroxyethylpiperazine-N'-ethanesulphonic acid

H^+-ATPase proton pumping ATPase

IgG immunoglobulin G

IgM immunoglobulin M

IP inositol phosphate

IP_2 inositol 1,4-bisphosphate

IP_3 inositol 1,4,5-trisphosphate

IP_4 inositol 1,3,4,5-tetrakisphosphate

ISA ionic strength adjuster

ITP inosine 5'-triphasphate

I_{50} concentration giving half maximal inhibition

LE mouse left ear

MES 2-(N-morpholino)ethanesulphonic acid

MOPS morpholinopropanesulphonic acid

M_r relative molecular mass

NAD nicotinamide adenine dinucleotide

NE mouse neither ear

NEM N-ethylmaleimide

nTP nucleotide triphosphate

PBS phosphate buffered saline

PBSA PBS with azide

PBSAT PBSA with Tween-20

PEG polyethyleneglycol

Pi inorganic phosphate

PI phosphatidylinositol

PIP phosphatidylinositol 4-phosphate

PIPK PIP kinase

PIP_2 phosphatidylinositol 4,5-bisphosphate

PMSF phenylmethylsulphonyl fluoride

poly polyclonal antiserum

QORase quinate: NAD^+ oxidoreductase

RE mouse right ear

R_f relative mobility

SDS sodium dodecyl sulphate (lauryl sulphate)

SDS-PAGE SDS-polyacrylamide gel electrophoresis

SR sarcoplasmic reticulum

TBS Tris-buffered saline

TCA trichloroacetic acid

TCM tissue culture medium

TEMED N,N,N',N'-tetramethylethylenediamine

Tris Tris(hydroxymethyl)aminoethane

Tween-20 polyoxyethylene sorbitol ester (polysorbate 20)

UTP uridine 5'-trisphosphate

Single letter amino acid abbreviations are IUPAC

Chapter 1

INTRODUCTION

Throughout the life cycle of higher plants, a range of environmental cues (such as gravity, nutrient status, temperature, quality, intensity and duration of illumination) and extracellular signals including plant growth substances, control diverse physiological and developmental processes such that ordered growth and development takes place in a manner which permits the plant to integrate with changing environmental conditions. The means by which such signals are perceived and transduced into an appropriate response is a major subject of interest. Environmental conditions have a much less profound effect on animal development than they do on plant development; research on the cellular mechanisms of control of growth and development in animals has therefore been concentrated on the mechanisms by which extracellular signals or hormones bring about appropriate cellular responses, and there is now extensive information available. Central to these mechanisms is the binding of the signalling molecule (usually a hormone) to a specific receptor molecule located in certain target cells. The receptor-hormone complex may be located in the cytoplasm and able to interact directly with DNA (after migration to the nucleus) to regulate gene expression and hence bring about a developmental response. An example of this type of mechanism are the steroid hormones (reviewed by Yamamoto, 1985; Gehring, 1987). These are small hydrophobic molecules

which pass through the plasma membrane of target cells; binding of the steroid to its receptor brings about a conformational change in the receptor molecule (receptor activation). The activated receptor has a high affinity for specific DNA sequences, and acts as a transcriptional enhancer, causing increased production of certain gene products. The gene products may in turn activate other genes to produce a delayed secondary response. A diversity of responses in different cells is made possible by the presence of different receptor proteins in certain cells, and by varying affinities of the activated receptor for different DNA sequences.

Similar mechanisms for control of developmental responses in plants via changes in gene expression occur, but less information is available as to the details of this process. It is clear that, for example, light regulates gene expression in several ways, and that light signals are perceived by photoreceptors such as phytochrome. Little is known about possible intermediate steps in transduction of the signal to bring about changes in gene expression (reviewed by Tobin and Silverthorne, 1985; Kuhlemeier *et al.* , 1987). Plant growth substances are also known to regulate gene expression (Guilfoyle, 1986; Theologis, 1986; Kuhlemeier *et al.* , 1987). For example, auxin affects many developmental responses (maintenance of apical dominance, xylem differentiation, cell elongation) and it has been suggested from studies on hypocotyl elongation that auxin may mediate two effects, a rapid response not involving gene expression and a long term process involving selective induction of genes. In most cases, the functions of auxin-regulated genes have not been elucidated, one exception being β-1,3-glucanase (Mohnen *et al.* , 1985). There is also evidence that high affinity membrane bound auxin-binding proteins, which probably function as receptors, are present in plant cells (Lobler and Klambt, 1985a,b). Van der Linde *et al.* (1984) isolated a soluble protein with high affinity for auxin from cultured tobacco cells which, when added to isolated tobacco nuclei, caused an auxin-dependent stimulation of transcription. It was not shown whether this effect was on specific auxin regulated genes, but the possibility of direct effects of growth substances

on gene expression was indicated. At present, therefore, the nature of mechanisms bringing about altered gene expression in plants in response to external stimuli are not clear.

The second way in which animal signalling molecules bring about cellular responses is via systems of intracellular messengers. Water-soluble signalling molecules (neurotransmitters, peptide hormones and growth factors) together with some lipid-soluble signalling molecules bind with high affinity to specific receptor proteins on the surface of target cells. Formation of the signalling molecule-receptor complex permits transduction of the extracellular signal into changes in the metabolic machinery of the cell via alterations in the levels of intracellular messenger compounds. Two of the most important intracellular messenger systems in animal cells involve cyclic nucleotides (cAMP, cGMP, cCMP, see reviews by Pastan, 1972; Sutherland, 1972; Goldberg and Haddox, 1977) and calcium ions (reviewed by Campbell, 1983; Rasmussen and Barrett, 1984). A common feature of the two pathways involving cAMP and calcium is that binding of the signalling molecule to its receptor alters the conformation of a cytoplasmic domain of the receptor which permits it to bind to a GTP-binding regulatory protein (G-protein, reviewed by Stryer and Bourne, 1986; Gilman, 1987). The G-protein then initiates a further chain of events involving cAMP or calcium by affecting plasma membrane enzymes or ion channels. In the intracellular messenger system involving calcium, activated G-proteins activate enzymes which, in turn, bring about formation of a soluble mediator, inositol 1,4,5-trisphosphate (IP_3), which causes release of calcium ions from intracellular stores, and diacylglycerol (DAG) which activates protein kinase C (reviewed by Sekar and Hokin, 1986; Bell, 1986; Nishizuka, 1986; Berridge, 1988a,b; Berridge and Irvine, 1989; see below for further details). These two major pathways interact in at least three ways (Cohen, 1988): (1) intracellular calcium and cAMP levels influence each other; (2) some calcium-dependent protein kinases (see below) are phosphorylated by cAMP- dependent kinases; (3) calcium-dependent and cAMP-dependent kinases may

phosphorylate different sites on the same protein.

The available evidence concerning the mechanisms of signal transduction involving intracellular messengers in higher plant cells suggests that a pathway involving calcium may be functional in plants (reviewed by Marmé, 1982, 1985, 1986; Marmé and Dieter, 1983; Hepler and Wayne, 1985; Gilroy *et al.* , 1987; Poovaiah and Reddy, 1987; Allan and Trewavas, 1987; Kauss, 1987; also volume edited by Trewavas, 1986). There is evidence for the presence of cyclic nucleotides in plant cells (Brown and Newton, 1981; Newton *et al.* , 1984), but as yet no good evidence for their physiological roles. Much research has been carried out within the last ten years to elucidate the role of calcium ions, diacylglycerol and inositol phosphates in the transduction of signals in plant cells. Of central importance in this pathway are changes in the concentration of cytoplasmic free calcium ($_p[Ca]_{cyt.}$). Calcium ions are suitable as intracellular messengers in such a system because of their unique properties (Levine and Williams, 1982). The atomic size, charge density, ionic radius and low free energy of hydration of calcium contribute to the fact that many macromolecules have a higher degree of selectivity for calcium than most other abundant cations (K^+, Na^+, Mg^{2+}). These physicochemical properties also result in greater restrictions for movement of calcium across membranes and greater affinity for binding to proteins in comparison with other more abundant ions. The maintenance of low cytoplasmic free calcium concentrations in unstimulated cells is an essential feature of the calcium/DAG/IP_3 intracellular messenger system in animals (see below) and a consequence of this is that formation of insoluble $Ca_2(PO_4)_3$ in the cytoplasm is prevented which permits cells to utilise high energy phosphate compounds. An additional feature of low $_p[Ca]_{cyt.}$ is that large changes in calcium concentration may be brought about by small changes in the absolute amount of calcium in the cytosol. This permits 100-fold changes in $_p[Ca]_{cyt.}$ to occur without disturbing the ionic environment of the cell and without the input of excessive amounts of energy to restore normal levels (see below).

Physiological process affected by calcium	Reference
Plant growth substance action:	
Auxin-induced elongation	Evans, 1984 (r)
Auxin-binding	Poovaiah and Leopold, 1976
Auxin transport	De La Feunte and Leopold, 1973
Auxin-mediated H^+ secretion	Cohen and Nadler, 1976
Cytokinin action	Elliot, 1980, 1983; Elliot *et al.*, 1983
Gibberellin action	Moll and Jones, 1981
Abscisic acid action	Owen, 1988 (r)
Gravitropism	Roux and Serlin, 1987 (r); Poovaiah *et al.*, 1987b (r)
Response to light	Hepler and Wayne, 1985 (r)
Abscission	Poovaiah and Leopold, 1973
Senescence and ripening	Ferguson, 1984 (r)
Physiological disorders	Bangerth, 1974 (r)
Cell polarity	Reiss *et al.*, 1986
Metaphase/anaphase transition	Hepler, 1986
Chromosome movement	Lambert and Vantard, 1986
Mimosa leaf movement	Toriyama and Jaffé, 1972
Circadian leaf movement	Satter and Galston, 1981 (r)
Guard cell swelling	De Silva *et al.*, 1985a, b
Enzymes:	
NAD kinase	Muto and Miyachi, 1977; Dieter and Marmé, 1980b
QORase	Ranjeva *et al.*, 1983
1,3-ß-glucan synthase	Kauss, 1987 (r); Paliyath and Poovaiah, 1988
Ca^{2+}-pumping ATPase	Dieter and Marmé, 1980c, d; 1981b

Table 1.1: Some examples of physiological and developmental events and enzymes regulated by calcium and/or calmodulin in higher plants.

The enzymes indicated are those affected by changes in cytoplasmic free calcium concentration.

(r) indicates a review article.

For protein kinases and enzymes/proteins regulated by phosphorylation, see Tables 1.3 and 1.4 respectively.

It has been suggested (Jaffé, 1980; Williamson, 1981) that if calcium is acting as an intracellular messenger the following criteria must be met:

1) Cytoplasmic free calcium levels must respond to external stimuli before the physiological response occurs.

2) Artificially inducing a change in $_p[Ca]_{cyt.}$ must initiate the physiological response.

3) A mechanism must be present within the cells by which changes in $_p[Ca]_{cyt.}$ can be sensed and changed into a physiological response.

4) Preventing the operation of the Ca^{2+} sensing system must block the physiological response.

Many physiological and developmental responses in plants have been suggested to involve calcium as an intracellular messenger, fulfilling all or some of the criteria above. These are summarised in Table 1.1, together with some enzymes regulated by calcium and / or the calcium-dependent regulator protein, calmodulin (see below)

Central to the role of free calcium as an intracellular messenger is the fact that its concentration in the cytoplasm is regulated. In unstimulated animal cells, $_p[Ca]_{cyt.}$ is maintained at 0.1 - 10 μM (reviewed by Carafoli, 1987). Measurement of such low levels of free calcium in the presence of millimolar levels of magnesium has presented technical problems (reviewed by Thomas, 1982) which are rendered more complex in plant cells by the presence of large vacuoles, chloroplasts and the cell wall (Thomas, 1985). However, some recent reports have provided measurements of $_p[Ca]_{cyt.}$ in higher plant cells. Gilroy *et al.* (1986) used Quin-2 to estimate $_p[Ca]_{cyt.}$ in mung bean protoplasts and recorded a value of 1.71 x 10^{-7} M; Bush and Jones (1987) used Indo-1 (Indo-1 and Quin-2 are both specific calcium sensitive fluorescent dyes) to record a $_p[Ca]_{cyt.}$ in barley aleurone protoplasts of 2.5 x 10^{-7} M; Felle (1988a and b) estimated $_p[Ca]_{cyt.}$ in *Zea mays* L. coleoptile and root hair cells to be in the range of 0.84 - 2.31 x 10^{-7} M using a calcium electrode. Gilroy *et al.* (1989) also published data using both Quin-2 and Aequorin (a Ca^{2+}-activated photoprotein) estimating $_p[Ca]_{cyt.}$ in carrot and barley protoplasts to be

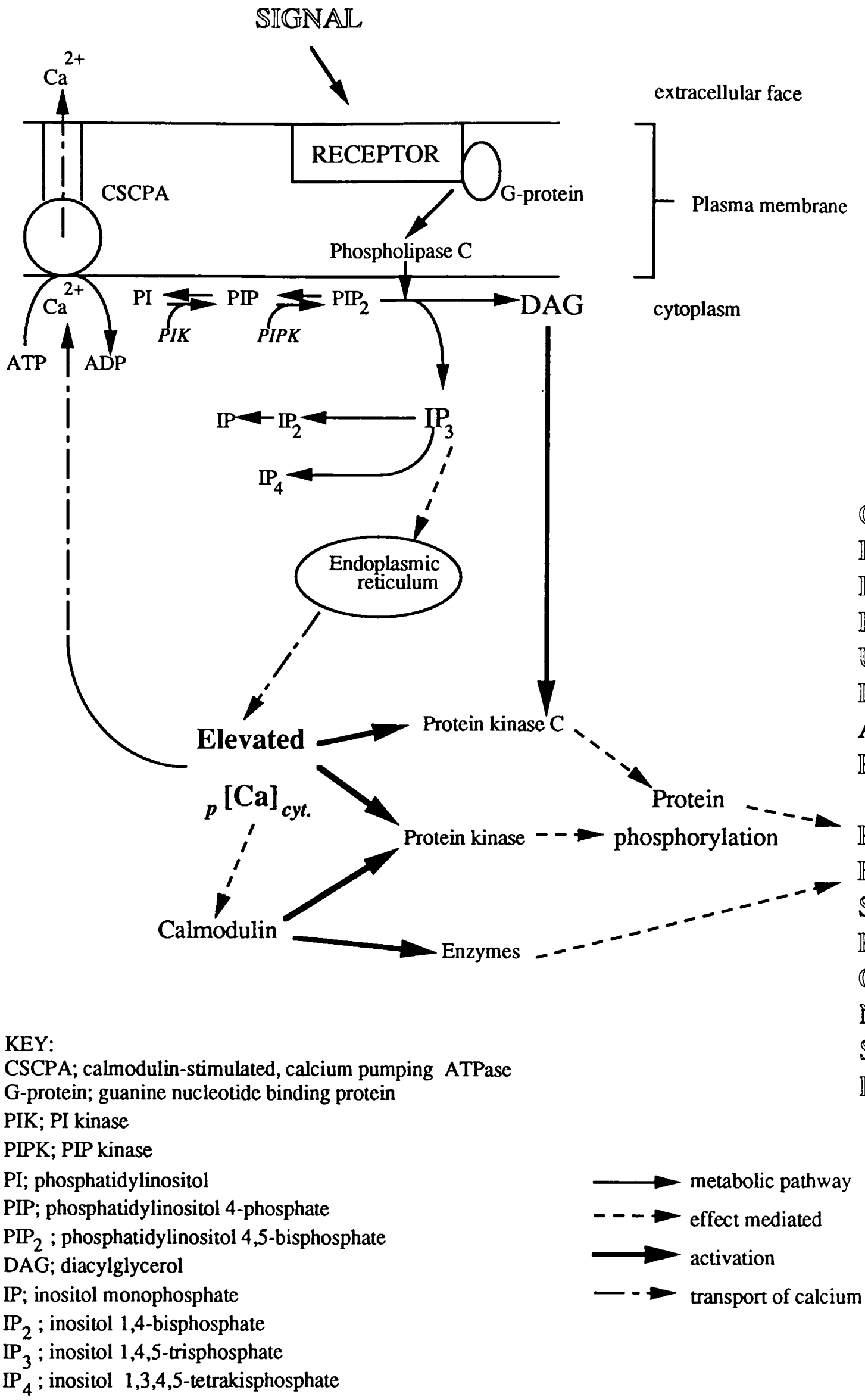


Figure 1.1: The calcium/inositol phosphate signal transduction pathway of mammalian cells.

Component of inositol phosphate transduction pathway	Presence in plants?	Physiological role?	Reference
Phospholipase C	yes	yes	Irvine *et al.*, 1980; Melin *et al.*, 1987; McMurray and Irvine, 1988
G-proteins	yes	?	Dillenschneider *et al.*, 1986; Blum *et al.*, 1988; Drøbak *et al.*, 1988b; Jacobs *et al.*, 1988
PI	yes	degraded after auxin treatment	Morré *et al.*, 1984; Heim and Wagner, 1987
phosphatidyl inositol kinases	yes	yes	Sandelius and Sommarin, 1986; Heim and Wagner, 1987; Heim *et al.*, 1987
PIP, PIP_2	yes	yes, amount PIP_2 varies with growth cycle	Boss and Massel, 1985; Heim and Wagner, 1986; Wheeler and Boss, 1987; Drøbak *et al.*, 1988a; Irvine *et al.*, 1989
DAG	yes	yes, amount varies with light but not auxin treatment	Morré *et al.*, 1989; Morse *et al.*, 1989b
IP, IP_2	yes	yes, turnover stimulated by light	Morse *et al.*, 1986; Morse *et al.*, 1987a, b; Rincon *et al.*, 1989
IP_3	yes	yes, induces calcium release from microsomes or protoplasts	Drøbak and Ferguson, 1985; Rincon and Boss, 1986; Reddy and Poovaiah, 1987; Morse *et al.*, 1987a; Rincon *et al.*, 1989
IP_4	--	--	--
Protein kinase C	yes	? similarity to mammalian enzyme	Elliot and Skinner, 1986; Elliot and Kokke, 1987; Elliot *et al.*, 1988
Protein kinases	yes	yes, but remains to be fully established for some types	see Table 1.3
Calmodulin	yes	see text	see text
Calcium- and calcium/calmodulin-regulated enzymes	yes	see Table 1.1	see table 1.1

Table 1.2: Evidence for presence and possible physiological role of compounds of inositol phosphate signal transduction pathway in higher plants.

Abbreviations as Figure 1.1.

in the range 1.3-3.6 x 10^{-7} M. These results are in close agreement with those made for animal cells.

These low $_p[Ca]_{cyt.}$ values are maintained despite high extracellular concentrations of calcium (1,000- to 10,000-fold that of the cytoplasm) which, together with the membrane potential (cytoplasm -120 to -170 mV) produces a large electrochemical gradient for calcium influx. Further evidence that $_p[Ca]_{cyt.}$ is regulated was provided by Felle (1988b) who showed that when the external concentration of calcium in the bathing medium surrounding maize coleoptiles was raised, there was a transient increase in $_p[Ca]_{cyt.}$ which was followed by a decrease to restore the original level. It was also shown (Felle, 1988a) that addition of 1 μM indole-3-acetic acid to the external medium surrounding epidermal cells of maize coleoptiles caused oscillations in $_p[Ca]_{cyt.}$, with a period of 20-30 minutes, as measured using a calcium-sensitive microelectrode. It was suggested that these oscillations originated from auxin-receptor complex-induced lipid breakdown and second messenger formation, perhaps via inositol trisphosphate / diacylglycerol. The lack of a sudden increase in $_p[Ca]_{cyt.}$ (which might be expected from the concept of calcium as a second messenger) was suggested to arise from the probable localised nature of the calcium bursts and unequal distribution of calcium in the cytoplasm.

The changes in $_p[Ca]_{cyt.}$ which are part of the stimulus-response pathway in plant cells are thought to be interlinked with other intracellular messengers, including diacylglycerol and IP_3 (reviewed by Morse *et al.* , 1989a, see also Table 1.2). In animal cells it is now well established that such a system is functional (reviewed by Berridge, 1988a,b). However, it is apparent that this system is very complex (Michell, 1986; Berridge and Irvine, 1989). Figure 1.1 summarises only the major features of it. It has been established that several of the components of this pathway are present in higher plant cells and have a physiological role; the evidence has been summarised in Table 1.2. It has also been shown in various studies that addition of auxins to plant cell membrane preparations preloaded with calcium causes release of the calcium (Buckout *et al.* , 1980, 1981).

Some of the effects of abscisic acid (ABA) on plant cells may also be mediated through changes in ${}_p[Ca]_{cyt.}$ (reviewed by Owen, 1988). Inhibition of stomatal opening by ABA is synergistically dependent on the presence of calcium (De Silva *et al.* , 1985a, b) and ABA and calcium have a synergistic effect on respiration in protoplasts from barley seedlings (Owen *et al.* , 1987). Calcium release from preloaded vesicles or protoplasts is also caused by addition of IP_3 to the assay (Drøbak and Ferguson, 1985; Rincon and Boss, 1987; Reddy and Poovaiah, 1987). A further control of ${}_p[Ca]_{cyt.}$ in animal cells occurs via a GTP-specific calcium releasing mechanism in a microsomal fraction, the effect of GTP being enhanced by the presence of IP_3 (Dawson, 1985; Dawson *et al.* , 1986, 1987). It is possible that GTP may also have a similar role in plant cells; Allen *et al.* (1989) showed that GTP caused release of calcium from pre-loaded microsomes from *Zucchini.*

Therefore it seems that various signals or components of the inositol phosphate signal transduction pathway can cause changes in ${}_p[Ca]_{cyt.}$. It has also been shown that auxin causes transient changes in the level of IP_3 and IP_2 (inositol bisphosphate) in cells of *Catharanthus roseus* L. (Ettlinger and Lehle, 1988) and a similar observation was recorded by Zbell and Walter (1988). Morse *et al.* (1987b, 1988) showed that phosphatidylinositol turnover was accelerated by light. Therefore, available evidence suggests that signal transduction in plants involving calcium may be interlinked with a pathway involving inositol phosphates and associated compounds (see below for discussion of protein kinases and protein phosphorylation in plants). However, there are lines of evidence suggesting differences between the inositol phosphate pathway in animals and the likely pathway in plants (Morse *et al.* 1989a) and "it is important for plant biologists to search for ...variations between the PI (inositol phosphate) cycle in plants and animals, rather than accepting the animal paradigm uncritically" (Morse *et al.* , 1989a).

The effects of changing levels of $_p[Ca]_{cyt.}$ on physiological and developmental processes are further modulated by calcium receptor proteins; in plants, calmodulin is the major such protein (reviewed by Dieter, 1984; Roberts *et al.* , 1986). Calmodulin is found in all eukaryotes and calmodulins from diverse sources show a high degree of structural and functional similarity. The first identification of calmodulin in plants was made by Anderson *et al.* (1980), and the amino acid sequence (Toda *et al.* , 1985) and cDNA sequence (Ling and Zielinski, 1989) of plant calmodulins show close similarity to animal calmodulin. Elevated levels of $_p[Ca]_{cyt.}$ cause calcium to bind to calmodulin, which brings about a large conformational change and increase in α-helix content of the calmodulin. The active calmodulin then interacts with an inactive or partially active enzyme, causing its activation. This may bring about the physiological response directly, or indirectly by protein phosphorylation. An additional level of control has been suggested in animal cells (Klee, 1980) to be in the formation of four calcium-calmodulin complex formations (depending on the number of calcium-binding sites occupied), to which different enzymes may be sensitive. Table 1.1 indicates physiological processes and enzymes which are regulated by Ca^{2+} and / or calmodulin, although in some studies, involvement of calmodulin has been deduced from effects of calmodulin antagonists, the use of which is subject to controversy. The presence of other calcium receptor proteins has also been suggested in plant cells (Chafouleas *et al.* , 1979; Van Eldik *et al.* , 1980; Ranjeva *et al.* , 1986) but the physiological significance of these has not been established.

As indicated above (Figure 1.1) the physiological response arising from signal perception and mediated by the probable calcium / inositol phosphate signal transduction pathway may also be brought about by protein phosphorylation which occurs in response to the activation of protein kinases (reviewed by Poovaiah *et al.* , 1987a). Various types of protein kinases have been identified in plants, as distinguished by their effector compounds (Table 1.3) and include a number of calcium and / or calmodulin regulated

Category of protein kinase	Reference
Messenger-independent	Ranjeva *et al.*, 1984
Calcium-dependent	Hetherington and Trewavas, 1982, 1984a Chiatante *et al.*, 1987 Harmon *et al.*, 1987 Polya *et al.*, 1987 Battey and Venis, 1988 Bogre *et al.*, 1988 Klucis and Polya, 1988 Schaller and Sussman, 1988 Feller, 1989 Oláh *et al.*, 1989 Putnam-Evans *et al.*, 1989
Calcium- and calmodulin-dependent	Polya and Davies, 1982 Polya *et al.*, 1983 Polya and Micucci, 1984 Blowers *et al.*, 1985 Blowers and Trewavas, 1987
Fatty acid-dependent	Klucis and Polya, 1987 Lucantoni and Polya, 1987
Phospholipid-dependent	Schafer *et al.*, 1985
Platelet-activating factor-dependent	Martiny-Baron and Scherer, 1988 Scherer *et al.*, 1988

Table 1.3 : Categories of protein kinase found in higher plants

protein kinases.

It has been shown recently that a phospholipid similar to platelet- activating factor (found in mammals and known to regulate various enzymes, reviewed by Hanahan, 1986) is present in plants and that it activates a protein kinase and a proton-translocating ATPase (Scherer 1985; Scherer and Stoffel, 1987; Martiny-Baron and Scherer, 1988; Scherer *et al.* , 1988). This line of study is one of several which indicate that plant cells may possess complex mechanisms of signal transduction in addition to the probable calcium /inositol phosphate pathway. The physiological role of many of these protein kinases remains to be fully established; however, the reports of Zocchi *et al.* (1983) and Zocchi (1985) which suggested that ~~protein~~ proton-pumping ATPase activity in microsomes was reduced by increased phosphorylation of membrane proteins were followed by the report of Schaller and Sussman (1988) who showed calcium regulated phosphorylation of serine and threonine residues of the plasma membrane proton pump of *Avena sativa* L. roots. Phosphorylation was stimulated by low concentrations of calcium and by a decrease in pH. These results were suggested to indicate that a calcium-dependent protein kinase mediated phosphorylation of the proton pumping ATPase, thus regulating its activity, and changes in $_p[Ca]_{cyt.}$ and pH were important in modulating this phosphorylation.

Protein phosphorylation and dephosphorylation are recognised as important mechanisms by which the activity of regulatory enzymes and receptor molecules is altered within animal cells in response to a wide range of stimuli (Cohen, 1988). General aspects of protein phosphorylation in plants have been reviewed by Trewavas (1976), Poovaiah and Reddy (1987), and Poovaiah *et al.* (1987). Calcium-promoted phosphorylation of soluble and membrane bound proteins has been shown in a variety of tissues from *in vitro* studies (Veluthambi and Poovaiah, 1984a); in some studies the role of calmodulin has also been implicated (Veluthambi and Poovaiah, 1984b). Raghothama *et al.* (1987) showed that *in vivo* protein phosphorylation in corn root tips was affected by manipulation of $_p[Ca]_{cyt.}$ and obtained some evidence that calmodulin might also be involved in

this process. However, it is evident that much further research in this area is required, especially in the identification of enzymes / processes regulated by calcium and / or calmodulin-dependent protein phosphorylation.

From this brief overview of the role of calcium in signal transduction and the probable role of inositol phosphates in plant cells, it can be seen that calcium has a central role. It seems likely that signal transduction in higher plants will involve more than just changes in free calcium levels and inositol phosphate metabolism as shown above (Figure 1.1). For example, Boustead *et al.* (1989) have demonstrated the presence of annexins in plant tissues. In animal cells annexins associate reversibly with acidic phospholipid bilayers in the presence of micromolar concentrations of calcium, (reviewed by Giesow *et al.* , 1987). Palmgren and Sommarin (1989) have shown that lysophospholipids stimulate activity of the proton-pumping ATPase from the plasma membrane of oat roots. Observations such as these, whilst of a preliminary nature at present, suggest that there may be several, possibly interlinked, pathways in which signals perceived by cells are transduced into appropriate cellular responses.

Calcium-dependent events are controlled in animal cells by two further mechanisms. Firstly regulation of calcium-sensitive components (whether direct or indirect) may occur by changes in calcium concentration (amplitude regulation) as indicated above. In plants, such changes may be long term (as in pollen tubes, Reiss and Nobiling, 1986) or transient (as is the case in cells undergoing mitotic progression, Keith *et al.* , 1986). Secondly, sensitivity regulation may occur in animal cells in which the affinity of the response element for calcium / calmodulin is altered. Evidence for the occurence of sensitivity regulation in plants is at present only indirect. For example, endogenous calmodulin inhibitors appear to decrease during germination (Cocucci and Negrini, 1988) and increase during senescence (Paliyath and Poovaiah, 1984).

It remains to be fully explained (for both animal and plant cells) how changes in $_p[Ca]_{cyt.}$ elicit a diversity of responses in different cells. The cell type and physiological

Response affected	Calcium-channel blocking agent	Reference
Auxin-induced coleoptile extension	Nifedipine	Hetherington and Trewavas, 1984b
Tip growth in pollen tubes	Nifedipine	Reiss *et al.*, 1986
Calcium entry into protoplasts	various	Graziana *et al.*, 1988
Binding to membranes/cells:		
pea seedling membranes	Nitrendipine	Hetherington and Trewavas, 1984b
zucchini membranes	Verapamil	Andrejauskas *et al.*, 1985

Table 1.4: Responses in higher plants affected by inhibitors of mammalian calcium channels

state of the cell (a result of differential gene expression) are important parts of this, as are the concentration and distribution of receptors for the primary signal. The amount, type and location of calcium binding proteins and the proteins they regulate are likely to vary between cells, as will inhibitors and calcium uptake / release mechanisms. Combinations of these features produce cells which are 'competent' to respond to extracellular signals and / or changes in $_p[Ca]_{cyt.}$ in defined ways. Cells at different stages in development will therefore vary in these features and hence respond in different ways to changes in $_p[Ca]_{cyt.}$. An additional point is that calcium has a low mobility in the cytoplasm (Gilroy *et al.* , 1987) raising the possibility of localised calcium regulated events within cells.

As indicated above, influx of calcium into plant cells is favoured by the large concentration and electrochemical gradient across the plasma membrane. The plasma membrane has a low permeability to calcium and hence represents a barrier to the transfer of calcium into the cytoplasm. There is evidence in animal cells that calcium influx occurs at the plasma membrane via calcium channels (Reuter, 1983), the action of which is controlled either by voltage-dependent gating, by ligands (eg neurotransmitters and hormones, Lee and Tsien, 1983) or in some cases by protein phosphorylation (Reuter, 1985). Evidence concerning the presence of calcium channels in plant plasma membranes is based on the effects of inhibitors of mammalian calcium channels on various cellular responses (Table 1.4). It has also been shown that calcium channel inhibitors bind to plant membranes (for example, nitrendipine, Hetherington and Trewavas, 1984; verapamil, Andrejauskas *et al.* , 1985). Unequivocal demonstration of the presence of calcium channels in plants awaits their purification and identification in reconstituted systems.

The study of mechanisms of calcium transport which result in efflux pumping from the cytoplasm, and hence maintenance of low $_p[Ca]_{cyt.}$, are an important area of research. In animal cells it has been shown that two main types of membrane calcium

transporters exist; calcium / cation antiporters and active (ATP-dependent) calcium pumps (reviewed by Rega and Garrahan, 1986). Investigations of the mechanisms of calcium transport in plant cells have involved study of the characteristics of calcium transporters in plant membrane preparations.

Biochemical characterisation of membrane ion transporters may be made by extracting a membrane fraction from the species of choice (see below) and then assessing the effects of various compounds, including ionophores, inhibitors and ions on transport and associated ATPase activities. Demonstration that ion transport is tightly coupled to ATPase activity (a primary transport mechanism) may be made by assessing the effects of ionophores and protonophores which dissipate ion and proton gradients. Antiport transport mechanisms will be inhibited in the presence of such compounds, but tightly coupled ion-pumping ATPases will not. Some of the ionophores used in studies of the proton tranlocating ATPase include nigericin (causes H^+/K^+ exchange), carbonylcyanide-p-trifluoromethoxyphenyl hydrazone, FCCP (a protonophore) and carbonylcyanide-m-chlorophenylhydrazone, CCCP (protonophore). The effect of these is to prevent the action of antiport systems by dissipating the ion gradient across the vesicle membrane (Sze, 1985 and references therein).

Before proceeding to consider evidence for the mechanisms of calcium efflux in higher plant cells, it is necessary to comment on the types of ATPase present in eukaryotic cells. These have been classified into three groups (reviewed by Pederson and Carafoli, 1987a and b) designated 'F', 'V' and 'P'. F-type ATPases are found in bacteria, chloroplasts and mitochondria and have a large molecular mass and complex subunit structure (reviewed by Futai *et al.* , 1989). The function of these ATPases in aerobic organisms is to synthesise ATP; this is carried out by a mechanism involving the electrochemical proton gradient which is suggested to involve phosphorylation of ~~ATP~~ ADP followed by induction of a conformational change bringing about ATP release. The subunits of F-ATPases have been divided into two categories, F_0 and F_1. F_0 subunits show the most variation in

FEATURE	H^+ PUMPING/ATPase[1]	
	TONOPLAST-TYPE	PLASMA MEMBRANE TYPE
Membrane origin	tonoplast and other (?ER)	plasma membrane and other (?Golgi)
Membrane density	1.10-1.13 g cm^{-3}	1.13-1.17 g cm^{-3}
Inhibitor:		
oligomycin (mitochondrial ATPase inhibitor)	NI	NI[a, g]
azide (mitochondrial ATPase inhibitor)	NI[l, m]	NI[f, g]
orthovanadate (inhibitor of P-type ATPases)	NI[b, l, m]	I_{50}=3-10 µM[b, c, d, f]
nitrate	I_{50}=10-15 mM[b, l, m]	NI[b, f]
DCCD (non-specific ATPase inhibitor)	I_{50}= 3 µM[k, m]	I_{50}=10-20 µM[c, e, f]
DES (non-specific ATPase inhibitor)	ND	inhibitory[a, c, f]
DIDS (anion transport blocker)	I_{50} = 5 µM[b, n]	varying effects[a]
NEM (specific, irreversible -SH group modifier)	I_{50} = 30 µM[n]	ND
Anion	stimulated[b, l, n]	little or no effect[b]
Cation	little or no effect[b]	stimulated[b, c, g]
Substrate specificity	ATP>GTP>NTP[n]	ATP>>NTP[c, d]
k_m (ATP)	0.1-0.2 mM[m, n]	0.3-0.5 mM[i]
pH optimum (ATPase)	8.0-8.5 (and 7.0-7.5)[j]	6.5-7.0[e, f]
Molecular weight	400-500,000 M_r[k]	100-105,000 M_r[c, d]
catalytic subunit	70,000 M_r[k]	?
other subunits	60,000 and 16,000 M_r[k]	approximately 60,000 and 70,000 M_r[g, h]

Table 1.5: Distinguishing properties of electrogenic, H^+ translocating ATPases associated with the tonoplast and plasma membrane of higher plant cells.

[1] The properties of the tonoplast and plasma membrane ATPase reflect a combination of ATPase and H^+ pumping activities unless otherwise indicated.

NI = not inhibitory
ND = not determined

Based on Sze (1985); references as in Sze (1985), additional references as indicated:

[a] Dupont and Leonard (1980)
[b] Churchill *et al.* (1983)
[c] Vara and Serrano (1982)
[d] Clement *et al.* (1986)
[e] Kasamo (1986)
[f] Anthon and Spanswick (1986)
[g] Kasamo (1987)
[h] Rausch *et al.* (1987)
[i] Brauer and Tu (1989)
[j] Henry and Pilet (1988)
[k] Mandala and Taiz (1986)
[l] Mandala and Taiz (1985)
[m] Manolson *et al.* (1985)
[n] Randall and Sze (1986)

the organisms studied, and are probably involved in energy coupling. The F_1 subunits contain high energy binding sites for ATP, forming a high energy F_1-ATP complex. Much research has been carried out concerning the reaction mechanisms of F-ATPases, current opinion (Pederson and Carafoli, 1987b) suggesting that energy coupling is indirect, tightly bound ATP synthesised by the ATPase being released by conformational changes induced by proton binding.

V-type ATPases (reviewed by Sze, 1985; Rudnick, 1986; Rea and Sanders, 1987) are located on membranes of organelles other than mitochondria and sarcoplasmic / endoplasmic reticulum. Three main tentative generalisations can be made concerning these ATPases: (1) they translocate protons; (2) they appear not to form covalent phosphorylated intermediates as part of the catalytic reaction cycle; (3) they are comprised of multiple subunits and have high M_r values.

Of most interest for direct calcium transport mechanisms are the P-type ATPases. The reaction pathway of these is characterised by the formation of a covalent phosphorylated intermediate which is formed when the γ-phosphate of ATP reacts with a single aspartic acid residue (see Chapter 5, section 5:1 for further details and references).

As indicated above, demonstration of ion-translocating ATPases involves the use of inhibitors and ionophores. Table 1.5 summarises the evidence concerning V- and P-type proton translocating ATPases in plant cells; this information provides a framework for the studies on active calcium-transporting systems described below. Export of free calcium from the cytoplasm may occur at various membrane sites (reviewed by Evans, 1988). These have been characterised to varying extents using the approaches outlined above. Mitochondria are capable of calcium uptake but this is thought not to be involved in regulation of ${}_p[Ca]_{cyt.}$ since mitochondrial affinity for calcium is at least ten-fold lower than that of microsomal membranes (Dieter and Marmé, 1983). The presence of a calcium-transporting ATPase at the endoplasmic reticulum (ER) of plants has been demonstrated. This transporter has a high affinity for ATP, is not inhibited by azide,

Property	Probable location of calcium transporter	
	Endoplasmic reticulum	Tonoplast
k_m Ca^{2+} transport (μM)	2.5[c]	42[a]
V_{max} Ca^{2+} transport (nmoles min^{-1} (mg protein)$^{-1}$)	0.13[c]	360[a]
pH optimum	7.2-7.5[c, h, i]	7.0-7.5[a]
Inhibited by ionophores/protonophores?	No[b, c, d, e, h,]	Yes[d, f, g, j, k]
Inhibited by vanadate?	Yes[d, e, h, i]	No[d]
Inhibited by nitrate?	No[d, e, h, i]	Yes[d, k]
Effect of calmodulin	None[c]	ND
Mg-ATP as substrate?	Yes[b, d, h, i]	Yes, for H^+ ATPase[j]
LIKELY NATURE OF TRANSPORTER	Direct calcium pump	Ca^{2+}/nH^+ antiporter

Table 1.6: Summary of properties of endoplasmic reticulum and tonoplast calcium transporting systems in higher plants.

References:

[a] Blumwald and Poole, 1986

[b] Buckhout, 1983

[c] Buckhout, 1984

[d] Bush and Sze, 1986

[e] Bush *et al.*, 1989

[f] Butcher and Evans, 1987a

[g] Butcher and Evans, 1987b

[h] Giannini *et al.*, 1987a

[i] Lew *et al.*, 1986

[j] Schumaker and Sze, 1985

[k] Schumaker and Sze, 1986

is sensitive to vanadate and insensitive to nitrate. This pump therefore has similar properties to the plasma membrane calcium pump (see below). One feature which may be characteristic of the ER pump and distinguish it from the plasma membrane calcium pump is that it appears not to be calmodulin sensitive (Buckhout, 1984). In addition the ER pump has an apparent molecular mass of about 96,000 M_r (Giannini *et al.* , 1987a), which is closely similar to that of the mammalian SR calcium pump (Hasselbach and Waas, 1982), whereas the plasma membrane calmodulin-stimulated calcium pump of mammalian tissues has a significantly higher M_r (134,683, Verma *et al.* , 1988). Studies on the M_r of the higher plant CSCPA are reported in Chapter 4. Export of calcium from the cytoplasm may also occur at the tonoplast membrane. It has been shown in most studies that tonoplast calcium transport is brought about by a Ca^{2+}/H^+ antiport mechanism (see Table 1.6 for references).

The main distinguishing features of this type of calcium transport are sensitivity to protonophores and strong inhibition by nitrate. There is one report of a direct calcium pumping ATPase at the tonoplast (Fukumoto and Venis, 1986). It is possible either that apple fruit differ from other tissues in this respect or that the studies were carried out using inadequately purified membranes, so the significance of this report is not clear. The properties of tonoplasts Ca^{2+}/H^+ antiport transport are summarised in Table 1.6.

However, ER and tonoplast calcium transporters have only a finite capacity for the sequestration of calcium, and so the plasma membrane is likely to be the major site for overall regulation of ${}_p[Ca]_{cyt.}$. Macklon (1984) used compartmental analysis of isotope fluxes in onion cells to show that a much larger calcium flux occurred across the plasma membrane than the tonoplast. There is strong evidence for the presence of a plasma membrane calcium transporting ATPase in higher plants which may be stimulated by calmodulin, the properties of which are considered in detail below. There is no evidence in plant cells for the presence of a low affinity, high capacity Na^+/Ca^{2+} exchanger which mediates export of free calcium from mammalian cells, together with a calcium pumping

ATPase.

As indicated above, plant cells contain several types of calcium transporting systems located on different membranes. Many of the studies on active calcium transport *in vitro* (which is presumed to represent calcium efflux from the cytoplasm) have been carried out using 'microsomal' vesicle preparations. It was originally suggested (Gross and Marmé, 1978) from differential and sucrose density gradient centrifugation that such preparations did not include mitochondria, intact etioplasts, endoplasmic reticulum and Golgi vesicles. The data for this supposition was not presented. Despite this, the assumption has been generally made that such mixed vesicle preparations (which are prepared by a variety of methods) are plasma membrane enriched, although it is possible that such a membrane fraction may contain a variety of membrane types. It is possible to distinguish plant membrane types by several methods. The biochemical markers which are associated with plasma membrane are K^+-ATPase (Hodges *et al.* , 1972) and glucan synthetase II (Van der Woude *et al.* , 1974). Binding of N-1-napthylphthalamic-acid (Lembi *et al.* , 1971) is also a feature which permits identification of plasma membranes. A morphological marker used to identify plasma membrane is staining by phosphotungstic acid (Nagahashi *et al.* , 1978), but the specificity of this stain is not absolute. In order to establish the presence of plasma membrane in a cell fraction it is necessary to use at least two of these means of identification.* The evidence for ATP-dependent calcium transport and related ATPase activity will therefore be considered in two sections, that associated with microsomal vesicles and that with plasma membrane vesicles purified by various methods and for which evidence of purity has been presented.

* A more recent method for establishing the purity of a plasma membrane fraction is to assay for vanadate sensitive ATPase activity (a marker for plasma membrane) and nitrate-sensitive ATPase activity (a marker for tonoplast). See Table 1.5.

1.1 Studies on calcium uptake and ATPase activity using 'microsomal' preparations

The first report of ATP-dependent calcium uptake in plants was made by Gross and Marmé (1978). Microsomal vesicles were prepared by differential centrifugation or centrifugation over a sucrose cushion from a variety of species, most of the data being obtained using dark grown *Zea mays* L. coleoptiles. Calcium uptake was measured by accumulation of $^{45}Ca^{2+}$, the vesicles being separated from the medium by filtration on 0.45 μm nitrocellulose membrane filters. This method was used in all the studies on calcium uptake in microsomal vesicles. The amount of ATP-dependent calcium uptake varied between 0.05-0.4 nmol min^{-1} (mg protein)$^{-1}$ according to the species. Calcium uptake by microsomes showed the following properties:

(a) Uptake was dependent on ATP; highest specificity was for ATP, although with ADP the activity was 86% of that in the presence of ATP.

(b) In the presence of 1 mM EGTA, calcium uptake was abolished.

(c) Calcium uptake was dependent on the presence of magnesium, and showed a pH optimum of 7.2

(d) Preloaded vesicles released calcium after treatment with calcium ionophores.

(e) Oxalate prolonged the period over which the vesicles took up calcium. The effect of oxalate on calcium uptake in zucchini was also reported by Gross (1982).

These basic features of microsomal calcium uptake were confirmed and extended by Dieter and Marmé (1980c) where uptake was shown to be maximal in microsomes from 4.5 day old dark grown maize seedlings. This may be a result of developmental regulation of the calcium transporter. Further indication that such regulation may take place was presented by Paliyath and Thompson (1988) where senescence-related changes in ATP-dependent calcium uptake by microsomes from carnation petals were demonstrated.

Further evidence for ATP-dependence of calcium uptake into microsomal vesicles was

presented by Schumaker and Sze (1985). In microsomal vesicles from oat roots, maximal calcium uptake (7 nmol (mg protein)$^{-1}$) was attained after 30 minutes incubation. ATP-dependent uptake was dissipated by addition of A23187 (5 μM final concentration) to preloaded vesicles. Sodium orthovanadate (200 μM) inhibited calcium uptake by only a small amount (8% inhibition of control with ATP) but 10 mM nitrate inhibited calcium accumulation by approximately 80% . These results probably reflect a predominance of tonoplast calcium transport activity in the microsomes. Reddy and Poovaiah (1987) showed ATP-dependent calcium uptake (15 nmol (mg protein)$^{-1}$ after 40 minutes) in microsomes from dark grown maize coleoptiles which was dissipated by 2 μM A23187 and inhibited (70%) by 1 mM orthovanadate. Microsomal vesicles prepared from *Pisum sativum* L. roots by Butcher and Evans (1987a and b) took up a total of 2.0 nmol calcium (mg protein)$^{-1}$ over 20 minutes, which was dissipated by addition of A23187 (5 μM final concentration). This report also showed that both calcium uptake and ATPase activity in microsomes were dependent on magnesium. Orthovanadate (20 μM) inhibited calcium uptake to about 30% of control and diethylstilbestrol (20 μM) inhibited all but 10% of the uptake. Addition of 50 mM KNO_3 to assays for calcium transport resulted in a 20% inhibition. The protonophore FCCP (20 μM) inhibited ATP-dependent calcium uptake by about 30% and nigericin (10 μM) inhibited 40% of the uptake. These properties are indicative of calcium pumping ATPase(s) together with a Ca^{2+}/H^{+} antiporter, this being confirmed by studies using purified membranes (Butcher and Evans, 1987b).

Several reports have suggested that microsomal calcium uptake may be modulated by a number of effectors. Probably the most important modulator of activity of this process is calmodulin. In early reports Dieter and Marmé (1980a, d, 1981a) demonstrated that calmodulin-stimulation of calcium uptake occured in microsomes from dark grown maize coleoptiles. The calmodulin-stimulated component of calcium uptake was 0.1-0.3 nmol Ca^{2+} min^{-1} (mg protein)$^{-1}$. In dark grown tissue (Dieter and

Marmé, 1983) the addition of calmodulin increased the affinity and maximal velocity of the calcium-transporting system (K_m-calmodulin = 22 μM, +calmodulin = 5.2 nmol min^{-1} (mg protein)$^{-1}$). Fluphenazine, an antipsychotic drug known to inhibit calmodulin-dependent processes, was shown to inhibit calmodulin-stimulated uptake (Dieter and Marmé 1980d). In the report by Dieter and Marmé (1983) full inhibition of the calmodulin-stimulated component was achieved at 20 μM fluphenazine, a concentration which also slightly inhibited calmodulin-independent calcium uptake. Zocchi and Hanson (1983) demonstrated calmodulin-stimulated ATP dependent calcium uptake (calmodulin-stimulated component 0.4 nmol min^{-1} (mg protein)$^{-1}$) in a sealed vesicle fraction from dark grown *Zea mays* L. roots. This evidence suggests that calmodulin interacts directly with the calcium pump to stimulate its activity. However, the effects of calmodulin antagonists on calcium transport of plant membrane preparations are not always straightforward, and such data must be interpreted with care (Trewavas, A., pers. comm.).

It has also been suggested that light modulates calcium transport by microsomal membranes. An early study by Gross *et al.* (1979) showed that calcium transport in microsomal membranes from etiolated corn was inhibited by white light. This was shown to be due to artefactual contamination of the membrane by protochlorophyllide$_{630}$. Later studies (Dieter and Marmé, 1981a, 1983) showed that far-red light affected calcium transport, again in microsomal preparations. Irradiating tissue for periods ranging from 6 hours to 5.5 days with continuous far-red light resulted in inhibition of calmodulin-stimulated calcium transport. Both the K_m and V_{max} for calcium were altered, as were basal (non-calmodulin-stimulated) levels, as indicated below (values for K_m in μM and for V_{max} in nmoles min^{-1} (mg protein)$^{-1}$):

Tissue	K_m		V_{max}	
	+CaM	-CaM	+CaM	-CaM
Dark grown	22	15	2.7	5.2
Far-red grown	27	26	2.3	2.8

Irradiation of maize microsomes with far-red light after preparation did not have any effect on either basal or calmodulin-stimulated calcium transport, indicating that the effect of far-red light was unlikely to be direct. The long periods of irradiation with far-red light required to obtain effects also suggest that a direct phytochrome response was unlikely in this instance.

Stosic *et al.* (1983) reported calmodulin stimulated calcium uptake in a microsome fraction from light grown spinach leaves. Microsomes prepared from whole leaves had a rate of calcium uptake of 0.05 nmol min^{-1} (mg protein)$^{-1}$. There appeared to be variation in the rates of calcium uptake in microsomes from different parts of the leaf, calmodulin-stimulated uptake in microsomes from the lower epidermis being greatest (0.11 nmol min^{-1} (mg protein)$^{-1}$) and that in microsomes from the lamina lowest (0.02 nmol min^{-1} (mg protein)$^{-1}$). It seems clear that it is possible to demonstrate calmodulin-stimulated calcium transport in both light and dark grown tissue. The effects of far-red light and the relevance of these *in vivo* require considerable further investigation. The effect of growth substances as modulators of microsome calcium transport is discussed in Chapter 7.

Calmodulin-stimulated ATPase activity in microsomes from dark grown maize coleoptiles was shown by Dieter and Marmé (1981b). In the absence of calmodulin, the specific activity of ATP hydrolysis was 49 nmol min^{-1} (mg protein)$^{-1}$, and with calmodulin

52 nmol min^{-1} (mg protein)$^{-1}$. This demonstration of calmodulin-stimulation of ATPase activity in microsomes permitted Dieter and Marmé (1981b) to partially purify calmodulin-stimulated ATPase activity by means of calmodulin-affinity chromatography (see Chapter 4, section 4:1 for further details). Zocchi and Hanson (1983) reported a much higher level of calmodulin-stimulated ATPase activity in a sealed vesicle fraction from corn roots (calmodulin-stimulated component 30 nmol min^{-1} (mg protein)$^{-1}$). The report of Oláh *et al.* (1983) suggested that treatment of wheat seedlings with benzylaminopurine (a synthetic cytokinin) increased the affinity of microsomal Ca^{2+}-ATPase activity for calcium and calmodulin; this is considered further in Chapter 7.

1.2 Calcium transport and associated ATPase activity in purified plasma membrane preparations

Several studies on calcium transport and ATPase activity of purified plasma membrane fractions have shown essentially the same characteristics of activity as those described for microsome fractions above. The majority of studies carried out have concerned calcium transport activity (only two reports having been published concerning related ATPase activity in purified fractions). Rasi-Caldogno *et al.* (1982) separated pea internode membranes into two fractions after metrizamide-sucrose discontinuous gradient centrifugation, 'heavy' (plasma membrane enriched) and 'light' (probably plasma membrane and tonoplast). The 'heavy' fraction was insensitive to FCCP, saturated by 8-10 mM $MgSO_4$, and could utilise ITP. This was interpreted as being equivalent to the system described by Dieter and Marmé (1980c). Discontinuous sucrose density gradient centrifugation was used by Kubowicz *et al.* (1982) to partially purify plasma membrane vesicles from etiolated *Glycine max* L. seedlings, in which ATP-dependent calcium uptake was demonstrated. In addition, pretreatment of hypocotyl segments with indoleacetic acid increased calcium uptake by 100% , whilst treatment with cytokinins was inhibitory.

Giannini *et al.* (1987b) purified plasma membrane fractions from *Beta vulgaris* L. storage tissue using a discontinuous sucrose density gradient, and demonstrated ATP-dependent calcium uptake. This was assigned to the plasma membrane because of its properties and because inhibitors were included in the assay to prevent calcium uptake by mitochondria and tonoplast. The calcium uptake was inhibited by 100 μM vanadate (75% inhibition), affected by N,N'-Dicyxclohexylcarbodiimide (75% inhibition) and diethlystilbestrol (DES, 40% inhibition) and showed greatest nucleotide specificity for ATP. The uptake was dependent on calcium, becoming maximal at 10 μM, and was also dependent on the magnesium-ATP concentration. These properties were as expected for an ion transporter located at the plasma membrane.

Rasi-Caldogno *et al.* (1987) used a continuous sucrose density gradient to purify plasma membrane from *Raphanus sativus* L. seedlings, and found the properties of ATP-dependent calcium uptake were essentially similar to those presented by Giannini *et al.* (1987b). Neither of these studies reported an effect of calmodulin on calcium transport. An extensive study of calcium transport by purified plasma membranes from pea roots was carried out by Butcher and Evans (1987a and b). Membranes were purified using a rapid sucrose-density-gradient technique, and marker enzyme analysis used to characterise the membrane fractions. In plasma membrane-enriched fractions the pH optimum for calcium uptake was 7.5-7.9. The highest initial rate of calcium uptake was obtained with 1 mM ATP (100%), but uptake also occurred with 1 mM GTP (50%), 1 mM ITP (40%) and 1 mM ADP (20%). The characteristics of this uptake were then further evaluated (Butcher and Evans, 1987b). Calcium uptake by plasma membrane-enriched fractions was inhibited by 100 μM vanadate by at least 90% ; nitrate (50 mM) and FCCP (20 μM) did not affect plasma membrane calcium transport. However calmodulin-stimulation of calcium uptake in the plasma membrane fraction was not demonstrated, nor were any effects of calmodulin antagonists.

Zocchi (1988) described the use of a discontinuous dextran-sorbitol gradient to produce plasma membrane enriched vesicles from corn roots which showed calmodulin-stimulation of calcium uptake (calmodulin-stimulated component 0.1 nmol min^{-1} (mg protein)$^{-1}$). Free-flow electrophoresis was used by Malatialy *et al.* (1988) to prepare a plasma membrane enriched fraction from light grown *Spinacia oleracea* L. leaves. ATP-dependent calcium uptake was demonstrated (about 6 nmol (mg protein)$^{-1}$ over 40 minutes at 10 μM added calcium). This uptake was increased by the addition of calcium to the medium, becoming maximal at 50 μM added calcium. Magnesium was required for uptake, and it was shown that uptake associated with plasma membrane was more than doubled by addition of 5 μg/ml calmodulin. In this preparation 50 μM vanadate completely inhibited calcium uptake, and addition of A23187 after loading caused complete loss of calcium from the vesicles. Nitrate (5 mM) and CCCP (10 μM) had only small or negligible effects on uptake.

Calcium uptake has been studied in sealed plasma membrane vesicles prepared by aqueous two-phase partitioning from leaves of the calcifuge plant *Commelina communis* L. (Graf and Weiler, 1989). The membranes were extensively characterised by marker enzyme analysis. Uptake of calcium in the presence of ATP was 5 nmol (mg protein)$^{-1}$ over 10 minutes; this was dissipated by addition of A23187. Calcium uptake was dependent on ATP concentration, becoming maximal at 1 mM ATP. The concentration of free calcium in the assay medium also affected the calcium uptake activity, this being greatest at 12 μM calcium. The pH optimum of uptake was 7.2. A very high concentration of orthovanadate (500 μM) only inhibited calcium uptake to 50% of control. Maximal calcium uptake occurred with ATP (100%) but uptake with GTP and ITP was about 70% . Lanthanum strongly inhibited calcium uptake with an I_{50} of 10 μM, and the most effective inhibitor out of the range tested was erythrosin B (I_{50} 100 nM). Calmodulin stimulation of calcium uptake activity could not be demonstrated in this study, and although trifluoperazine (0.1 mM) was inhibitory, this inhibition was not overcome by

addition of calmodulin. The presence of calcium-stimulated ATPase activity in plasma membrane enriched vesicles purified by sucrose density gradient centrifugation from maize coleoptiles was demonstrated by Carter and Tipton (1987). In this study, the pH of the assay medium used was 6.5; most other studies have shown that this pH is optimal for activity of the proton-pumping ATPase rather than the calcium-pumping ATPase. The ATPase activity of the membrane bound enzyme was shown to be stimulated by calcium at physiological levels. Calmodulin was reported to stimulate activity by 30% , and fluphenazine was reported to inhibit 60% of the calcium-stimulated activity. This calcium-stimulated ATPase activity was not affected by oligomycin or sodium azide; DES and DCCD partially inhibited the calcium-stimulated component, but sodium molybdate and sodium orthovanadate affected basal, not calcium-stimulated activity. Purification of the ATPase was undertaken using a combination of hexyl agarose, ion exchange and gel permeation chromatography. No data on relative molecular mass ~~was~~ were presented and it was noted that the enzyme was only active in the absence of added magnesium (unlike that described by Dieter and Marmé, 1981b). In view of the very different assay and purification conditions used, it is not clear whether the $(Ca^{2+}+Mg^{2+})$-ATPase reported by Carter and Tipton is equivalent to the calmodulin-stimulated ATPase reported by Dieter and Marmé (1981b), Robinson *et al.* (1988) and described in this thesis.

Calmodulin-stimulation of ATPase activity in a purified and well characterised plasma membrane fraction was described by Robinson *et al.* (1988). Plasma membranes were isolated by aqueous polymer two-phase partitioning from light grown leaves of *Zea mays* L. Calmodulin enhanced the ATPase activity, giving maximal stimulation at 1 μM (4.5 nmol min^{-1} (mg protein)$^{-1}$). Calmodulin-stimulated ATPase activity was maximal at pH 7.2, and was fully inhibited by the calmodulin antagonist W7 and 50% inhibited by 125 μM lanthanum.

From the information presented above, it can be seen that demonstration of mechanisms for calcium efflux from higher plant cells is of importance in increasing understanding of regulation of cytoplasmic free calcium levels, which then leads towards understanding of the role of calcium ions in signal transduction. At the onset of the studies described in this thesis, available evidence indicated the presence of a calcium-pumping ATPase (probably on the plasma membrane) in higher plant cells, the activity of which may be regulated by calmodulin. Unequivocal proof of the presence of such a calcium transporter required further purification, an objective which has been carried out in the studies presented here, together with characterisation of some of its properties.

Chapter 2

MATERIAL AND METHODS

2.1 MATERIAL

All chemicals (except where indicated in text) were obtained from BDH (Poole, Dorset, U.K.) or Sigma (Poole, Dorset, U.K.) and were of at least 'AnalaR' grade or equivalent. Sources of other material are indicated in text.

2.2 METHODS: Purification and assay of calmodulin-stimulated ATPase

2.2.1 Plant material

Zea mays L. (c.v. Golden Bantam, Booker Seeds Ltd., Boston Road, Sleaford, Lincolnshire, U.K.) seeds of known dry weight were surface sterilised in sodium hypochlorite (approximately 1% (w/v) available chlorine) for 20 minutes in the dark. The seeds were rinsed in five changes of distilled water, and soaked in distilled water with aeration in an incubator at 30±1°C for 24 hours in total darkness. After this period, the seeds were transferred under green safelight to either:

a) black plastic containers with stainless steel mesh supporting racks over distilled

water containing 0.1 mM $CaCl_2$ which was aerated, or b) plastic containers with perlite soaked in 0.1 mM $CaCl_2$ and 0.1% (w/v) 'Dithane 945' (Pan Britannica Industries Ltd., Waltham Cross, Herts., U.K.).

The tubs were sealed and kept for a further 3.5 days in the incubator.

2.2.2 Membrane isolation

Membrane isolation was based on the method of Dieter and Marmé (1981b), modified as follows. All operations were carried out at 4°C, all buffers were freshly made in distilled, deionised water, and solutions containing Triton X-100 were sonicated before use. Coleoptiles were harvested, and their fresh weight recorded. The coleoptiles were chopped into approximately 5 mm sections and then ground in a pestle and mortar in 2 ml: 1 g fresh weight of Buffer A (Basic Buffer of 10% (w/v) sucrose, 25 mM MOPS titrated to pH 7.5 with solid Tris, plus 5 mM EDTA). Half the required volume of Buffer A was added at first, the tissue homogenised for about 1 minute, the rest of the buffer added, the mixture rehomog then filtered through four layers of muslin. The filtrate was centrifuged at 6,000xg for 15 minutes (Sorvall RC5C, SS34 rotor). The supernatant (6KS) was centrifuged at 48,000xg for 20 minutes (Beckman L8-70M Ultracentrifuge with 60Ti rotor (parameter ω^2t set at $9.63x10^9$ rad^2 s^{-1}), or Sorvall TFT 50.38 ($\omega^2t=6.96x10^9$ rad^2 s^{-1}). The pellet (48KP1) was resuspended in a volume of Buffer B1 (as Buffer A, but without EGTA and with 1 mM DTT) equal to that of 48KS1, and centrifuged at 48,000xg for 20 minutes. The pellet from this centrifugation (48KP2) was resuspended in 2-10 ml of Buffer B1 or B2 (as Buffer B1, with 0.02% (w/v) Triton X-100) for use in calcium transport or ATPase assays respectively. The microsome preparation was kept on ice and routinely used in the enzyme assays within 1-2 hours. During membrane preparation, samples of each fraction were taken and assayed for protein content by a modified Lowry assay.

In some experiments, as indicated in the text, this method of membrane isolation was modified to include 1 mM PMSF. The PMSF was made up as a stock at 20 mg/ml

in ethanol, and was added freshly to the buffers.

2.2.3 Purification of calmodulin-stimulated ATPase from maize coleoptiles by calmodulin affinity chromatography

The method was based on that of Dieter and Marmé (1981b) and Niggli *et al.* (1979b). The washed microsome pellet (48KP2) was resuspended in 2-5 ml of Buffer B1 and membrane proteins solubilised with Triton X-100 (1 mg/mg protein) added from a stock solution of 10 mg/ml in Basic Buffer. The amount of protein in the washed microsome preparation was estimated by comparison with previous extractions, where the protein content had been determined by a modified Lowry protein assay, since rapid methods for estimating protein concentrations were open to errors resulting from the nature of the sample. The mixture was shaken vigorously at 4°C for 10 minutes on a Vortex evaporator (Searle Buchler Instruments, setting 9). Sodium chloride (2.5 M stock in distilled water) and $CaCl_2$ (100 mM stock in Buffer B1) were added to 0.5 M and 5 mM respectively. Unsolublised material was pelleted by centrifugation at 100,000xg for 30 minutes (Beckman L8-70M with 60Ti rotor ($\omega^2t=2.7x10^{10}$ rad^2 s^{-1}) or TFT50.38 rotor ($\omega^2t=2.18x10^{10}$ rad^2 s^{-1}). The supernatant (100KS) was applied to a calmodulin-agarose affinity column (Sigma phosphodiesterase 3':5'-cyclic nucleotide activator-agarose, 5 ml volume), which had been pre-washed with several bed volumes of Buffer C (Basic Buffer with 0.5 M NaCl, 5 mM $CaCl_2$ and 0.05% (w/v) Triton X-100). Buffer flow was provided by an EYELA microtube pump, flow rate 0.7-1.0 ml/minute. Fractions of 1-2 ml were collected from the time the 100KS fraction was loaded. The absorbance of the fractions at 280 nm was recorded using a Philips PU 8720 UV/VIS scanning spectrophotometer. After loading, the column was washed for a minimum of 2 hours with Buffer C; in some cases the time of washing was extended, as indicated in the text. This basic purification procedure was further modified as indicated in the text, to include the addition of 1 mg asolectin/ml of 100KS (the addition being made

from a concentrated stock of 20 mg/ml in Basic Buffer, sonicated vigorously before use). Asolectin (0.1% (w/v)) was also added to the column buffers; the phospholipid was thoroughly sonicated into the buffers, and the buffers were filtered before use.

After the appropriate wash with Buffer C, calmodulin-binding proteins were eluted by the addition of Buffer D (Buffer C without $CaCl_2$, but with 10 mM EGTA). The peak fractions were brought to 10 mM $CaCl_2$, and further processed as appropriate.

In experiments where purification was to be carried out in the presence of protease inhibitors, 1 mM PMSF was added freshly to all the buffers, and the column was washed for two hours.

2.2.4 Assays for ATPase activity

i) Spectrophotometric method (adapted from Scherer and Morré, 1978)

The assays were carried out in acid washed (0.1 M HC1 in distilled deionised water, followed by two rinses in deionised water) 'Eppendorf' tubes and scintillation vial inserts. Each assay point was performed in triplicate. The total assay volume was 500 μl, which contained appropriate volumes of the following, according to experiment:

a) Incubation medium (Basic Buffer with 5 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mg/ml BSA, $1x10^{-6}$ M oligomycin (stored at -20°C in ethanol at 5 mg/ml) and 0.02% (w/v) Triton X-100).

b) ATP (disodium salt, vanadate free) or other nucleotide di- or tri- phosphates freshly made up in incubation medium to 5 mM (using A_{280} values and extinction coefficients to set concentrations exactly). Nucleotides were included in the assays to a final concentration of 1 mM.

c) Calmodulin (Sigma, from bovine brain) was stored as a concentrated stock in calmodulin storage buffer (25 mM MOPS, titrated to pH 6.5 with solid Tris, with 1 mM $CaCl_2$ and 0.1 M NaCl) at 0.1 mg/ml. Aliquots of this solution were stored at -20°C, and remained active over 12-16 weeks. The activity of the calmodulin preparations was

verified by assaying for stimulation of calmodulin-free phosphodiesterase (Boehringer-Mannheim assay kit, used according to the manufacturers' instructions). Concentrated calmodulin was diluted appropriately into incubation medium and added to the assay tubes shortly before addition of membrane preparation.

d) Sodium orthovanadate (in incubation medium) $5x10^{-6}$ M to $1x10^{-4}$ M final concentration.

e) Membrane preparations, 50 μl of microsomes were added to each assay (0.1-0.25 mg protein per assay).

Appropriate blanks (without membrane preparation and without nucleotide) were set up each time the assay was carried out. Sample tubes were incubated on ice until all additions had been made; the membrane preparations were added last, the tubes shaken and transferred immediately to a shaking water bath at 37°C for 30 minutes. Care was taken with the microsomal samples to ensure that they were evenly suspended, since the addition of varying amounts of membrane preparation (although the same volume was added) was one of the major sources of error in these experiments. Small groups of samples were processed at one time. The reaction was stopped with 1 ml of ice cold 10% (w/v) trichloroacetic acid (TCA) and kept on ice. Precipitated material was pelleted at 14,000xg (Sorvall RC5B centrifuge, SS34 rotor with inserts) for 5 minutes. From the supernatants, 500 μl was removed to scintillation vial inserts; to these, 1 ml of freshly prepared phosphate reagent (9 ml of 10% (w/v) ammonium molybdate and 4.5 ml of concentrated H_2SO_4 in 360 ml deionised water, plus 18 g of ascorbic acid) was added. The samples were mixed and incubated for 20 minutes in a water bath at 30°C. A standard curve was set up, using 0.05 - 0.5 μmoles of KH_2PO_4 in 250 μl deionised water with 250 μl 10% (w/v) TCA. The absorbance of the samples at 650 nm (Perkin Elmer Lambda 1 UV/VIS spectrophotometer) was recorded. A sample calibration curve is shown in Figure 2.1.

ii) Radioisotope method (based on the method of Seals *et al.* , 1987[78])

Assay tubes (scintillation vial inserts) were set up on ice in the same way as for the spectrophotometric assay using a total volume of 500 μl; three replicates of each point were set up. [γ^{32}P]-ATP (Amersham or New England Nuclear, adenosine 5'-triphosphate, tetra (triethylammonium) salt, 3000 Ci/nmol, 111 TBq/nmol) was added to unlabelled ATP to give a final concentration of 0.1-1.0 μCi and 1 mM unlabelled ATP per assay. Tubes without membrane or purified calmodulin-stimulated ATPase were preincubated for 5 minutes at 37°C in a vigorously shaking water bath. Samples (50 μl) were removed from the blanks (tubes with all additions except membrane or purified calmodulin-stimulated ATPase) and transferred to other scintillation vial inserts. Scintran Cocktail 'T' (4 ml) was added to these tubes to quantitate the total [γ^{32}P]-ATP added to each assay tube. The reaction was started by the addition of 50 μl of the various membrane or purified calmodulin-stimulated ATPase preparations. The tubes were shaken and incubated for a further 30 minutes in the water bath. To terminate the reaction, 90 μl of 6% (w/v) lauryl sulphate (SDS) was added.

Extraction of [γ^{32}P] was carried out by the addition of 300 μl of phosphate reagent (5 M H_2SO_4, 10% (w/v) ammonium molybdate, 0.1 M silicotungstic acid, mixed 2:2:1 by volume just before use), followed by 4.5 ml of 65% (v/v) xylene/35% (v/v) isobutanol. To minimise acid- and molybdate-catalysed ATP hydrolysis, this step was carried out with groups of 4-6 tubes at a time. The aqueous and organic phases were allowed to separate by letting the tubes stand for 5-10 minutes. An aliquot of the organic phase (1 ml) from each assay tube was removed to scintillation vial inserts and 4 ml of Scintran Cocktail 'O' was added. Samples were left overnight before they were counted in an LKB Rackbeta liquid scintillation counter in channels ratio mode. The counting efficiency was determined by comparison with standards; the efficiency of the phosphate extraction was 80% .

2.2.5 Protein assay

The modified protein assay of Bensadoun and Weinstein (1976), based on the method of Lowry *et al.* (1951) was used to allow for the presence of interfering materials.

Stocks of the reagents were made up as follows in deionised water and were stored at room temperature, except d), e) and f) which were kept at 4°C:

a) Lowry A - 0.5% (w/v) $CuSO_4$ in 1% (w/v) sodium citrate.

b) Lowry B - 2% (w/v) Na_2CO_3 in 0.1 M NaOH.

c) 24% (w/v) TCA.

d) 2% (w/v) deoxycholic acid (sodium salt).

e) BSA standard (Sigma, essentially fatty-acid free) at approximately 1 mg/ml, concentration determined using the A_{280} value and extinction coefficient.

f) Folin and Ciocalteu's phenol reagent (BDH).

Aliquots of the protein samples, at two or three different dilutions for each sample, were made up to 3.0 ml in plastic flat bottomed scintillation vial inserts. For this assay, a maximum of 0.18 mg of protein was added. A standard curve using the BSA stock was produced on each occasion that the assay was carried out. At each stage, the protein was resuspended using a Fisons Whirlimixer or Gallenkamp Spinmix. To solubilise the proteins, 25 μl of Na-deoxycholate was added; the samples were left to stand for 15 minutes at 4°C. TCA (1 ml) was added to precipitate the protein, which was pelleted at 2,000xg for 30 minutes at 4°C (MSE 'Mistral 4L', 4x480 ml swinging bucket rotor with inserts). The supernatant was removed by aspiration with a Pasteur pipette attached to a filter pump, leaving the pellet undisturbed in less than 50 μl of supernatant. The protein was redissolved in 1.5 ml of Lowry C (1 ml Lowry A and 50 ml of Lowry B mixed just before use), then 0.15 ml of diluted Folin-Ciocalteu reagent (diluted 1:1 with deionised water just before use) was added. The samples were left to stand in the dark for 45 minutes and the absorbance at 660 nm was determined (Perkin-Elmer Lambda 1 spectrophotometer of Philips PU 8720 UV/VIS scanning spectrophotometer). A sample

calibration curve is shown in Figure 2.2.

2.2.6 Reconstitution of purified calmodulin-stimulated ATPase

Reconstitution of purified calmodulin-stimulated ATPase into phospholipid vesicles was attempted using the method of Niggli *et al.* (1987), which is derived from the methods of Niggli *et al.* (1982) and Kagawa and Racker (1971).

Samples of purified calmodulin-stimulated ATPase were prepared using a 2 hour column wash with or without asolectin or PMSF in the column buffers. The EGTA eluted peak fractions were brought to 10 mM $CaCl_2$.

Asolectin (50 mg, from soybean, Sigma, L-α-phosphatidylcholine) was disolved in 1 ml diethylether and dried onto the walls of a small glass tube under nitrogen. The lipid was dispersed in 1 ml of medium containing 80 mM potassium cholate, 100 mM KC1, 20 mM HEPES, pH 7.2 at 4°C, 1 mM $MgCl_2$, 0.05 mM $CaCl_2$, and vortex mixed until all lipid was removed from the tube wall. This medium, and the dialysis medium, were made up in plastic vessels (washed to be calcium free) with ultrapure (essentially calcium-free) water.

A Dawe Soniprobe with fine tip was used to sonicate the asolection in the dispersal medium; this was carried out under nitrogen and on ice in 15 second on/off pulses. Sonication was continued until the suspensions were clear, or until no further change occured.

A sample (350 μl) of calmodulin affinity column purified extract (concentration 0.01-0.07 mg/ml) was added to the cholate/lipid mixture and gently mixed on a vortex mixer. The mixture was then dialysed in narrow dialysis tubing against three changes of dialysis buffer (130 mM KCl, 20 mM HEPES, pH 7.2, 1 mM DDT, 0.05 mM $MgCl_2$) for 16 hours at 4°C. Samples of the reconstituted calmodulin-stimulated ATPase were then assayed for ATPase activity (radioisotope assay).

2.2.7 Formation of phosphorylated intermediates

i) Assay method.

The method was based on that of Niggli *et al.* (1979b). Incubation medium was made up as follows: 130 mM KCl, 20 mM Tris, pH 7.4 with 1 M HCl, 12.5 μM $MgCl_2$. The following solutions were made up in incubation medium - final concentrations are indicated (in a total assay volume of 500 μl, made up to volume with incubation medium):

1 μCi/assay [γ^{32}P]-ATP.

5, 50 or 500 μM $CaCl_2$.

50 μM $LaCl_3$.

500 μM EGTA (solution brought to pH 8.0 in order to dissolve the EGTA).

50 nM calmodulin.

To 100 μl of either washed microsome sample (100-200 μg protein) or purified calmodulin-stimulated ATPase (2-10 μg protein), appropriate volumes of calmodulin, $CaCl_2$, $LaCl_3$ or EGTA were added, to give the concentrations above. In some experiments, the amount of [γ^{32}P]-ATP was increased to 5 μCi per assay, and 2 μM unlabelled ATP was also added. The reaction was started by the addition of the appropriate amount of [γ^{32}P]-ATP. The tubes were incubated for 15 seconds on ice; during this time they were briefly vortex mixed. Where cold ATP chase was used, 50 μl of 50 mM unlabelled ATP (giving final concentration of 5 mM) in incubation medium was added for 1 minute after the initial incubation. The reactions were terminated by pipetting the reaction mixture into 500 μl of stopping solution (10% (w/v) TCA, 0.1 mM ATP and 0.8 mM phosphoric acid) in an 'Eppendorf' tube treated with 'Repel-Silane' (BDH). The proteins were pelleted by centrifugation at 13,000xg for 5 minutes (Sorvall RC5B, with SS34 rotor and inserts for 'Eppendorf' tubes). The pellets were washed once without resuspension in 1 ml of deionised water. In some assays, 500 μl of 50 mM hydroxylamine monohydrochloride (in 100 mM MES-KOH, pH 6.0) was used in place of the water wash; in control assays, the pellet was washed in 500 μl of MES-KOH solution

only. An additional control was inclusion of boiled microsome preparation in the assay. Details of permeabilisation and solubilisation treatments are described in Chapter 5.

The final pellets were prepared for electrophoresis on Weber and Osborne (1969) pH 6.0 gels by taking up the pellet in a minimal volume (20 μl) of Weber and Osborne sample buffer. The samples were not boiled, but incubated on ice for a minimum of 30 minutes before loading onto the gel (see section 2:3). After electrophoresis, the gels were dried at 80°C for 1-2 hours (Bio-Rad model 483 slab dryer). Autoradiographs of the gels were then prepared (section 2:3:4).

ii) Estimation of free calcium in assays for phosphorylated intermediate formation.

This was carried out using a calcium sensitive electrode. The electrode was calibrated by additions of known calcium concentration to ultra pure water, with and without ionic strength adjuster (ISA). The change in mV reading over ten-fold concentration change was determined; a value of -25 mV was found both in the presence and absence of ISA. This value was close to the theoretical value of -27 mV stated in the electrode specifications. Known additions of calcium were then made to the incubation medium for the phosphorylated intermediate assays, again with and without ISA. The change in mV reading with ten-fold concentration change was determined, and these results compared with those from the calibration in water. With addition of ISA, the gradient was -22 mV, and without ISA was -28 mV. On this basis it was decided to use the calibration without addition of ISA as a means of estimating the free calcium concentration in the assay media. To do this, mock assays (without ISA) were made up in a volume of 10 ml, exactly as described above. Microsomes were replaced by Buffer B2, and purified calmodulin-stimulated ATPase by Buffer D with $CaCl_2$. The electrode readings obtained from these samples were converted into concentrations from the calibration curve of known additions of calcium into water without added ISA.

2.2.8 Localisation of calmodulin-binding proteins

Purified calmodulin-stimulated ATPase (prepared in the presence of protease inhibitors, and with a 2 hour column wash without phospholipid in the column buffers) was electrophoresed on 7.5% neutral SDS polyacrylamide gels as described in section 2:3. The polypeptides were electrophoretically transferred to nitrocellulose membranes as described in section 2:4:1. The membranes were blocked for 3 hours at room temperature with agitation in an excess of gold-calmodulin buffer (0.15 M NaCl, 0.01 M Tris, 5 mg/ml gelatin, at pH 7.0). Calmodulin-binding proteins were detected using colloidal gold labelled calmodulin (Sigma, 20 nm gold), diluted 100-fold in gold-calmodulin buffer. The membranes were incubated overnight, then washed three times with gold-calmodulin buffer before silver enhancing the gold signal with a Janssen silver enhancement kit, used according to the manufacturers' instructions.

2.3 METHODS: SDS polyacrylamide gel electrophoresis

The techniques were based on those described in Hames and Rickwood (1983).

2.3.1 Sample preparation

i) Laemmli (1970) gels. Samples of microsomal proteins or purified calmodulin-stimulated ATPase were concentrated (if required) either in a microconcentrator or by TCA precipitation. For microconcentration, samples of purified calmodulin-stimulated ATPase were centrifuged at 5,000xg for 30 minutes (Sorvall RC5B, SS34 rotor) in Centricon-10 microconcentrators (Amicon, Stonehouse, Gloucestershire, U.K.). This resulted in a reduction in volume of the sample of about 75% . Precipitation by TCA was carried out by the addition of an equal volume of stopping solution (section 2:2:7) to the sample. The mixture was incubated for 15 minutes on ice, and then centrifuged for 15 minutes at 13,000xg. The pellet was washed once with washing solution (section 2:2:7) and once with deionised water.

Samples for electrophoresis were then taken up in SDS-sample buffer (aliquots of single or double strength sample buffer being kept at -20°C; the final concentrations of the components were - 0.0625 M Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (w/v) glycerol, 0.01% (w/v) bromophenol blue, 10 mM DTT. If required, PMSF (5 mM) and chymostatin (500 μg/ml) were added to the sample buffer just before use. These samples were incubated on ice for at least 30 minutes. When protease inhibitors were not included, the samples were incubated in a boiling water bath for 3 minutes. In all cases, unsolubilised protein was pelleted by centrifugation at 13,000xg for 2 minutes (Beckman 'Microfuge B' or Dupont Sorvall 'Microspin 12S'). Up to 100 μg of complex protein mixtures and 10 μg of each polypeptide were loaded into the wells (8 mm) of 7.5% or 5% gels. Relative molecular mass markers (Sigma, high molecular weight standard mixture), were made up in single strength sample buffers and prepared as above by boiling and centrifugation.

ii) Sample preparation for Weber and Osborne (1969) gels. The pellet from the final wash of the assay for phosphorylated intermediate formation (section 2:2:7) was resuspended in a minimal volume of 100 mM sodium phosphate, pH 6.0, 1% (w/v) SDS, 10% (w/v) glycerol and 0.002% (w/v) bromophenol blue. If required, protease inhibitors were added to the sample buffer just before use as described for the Laemmli gels; the samples were then further processed as described above (section 2:3:1i). Relative molecular mass marker proteins (Sigma, high molecular weight standard mixture) were made up in Weber and Osborne sample buffer as above.

2.3.2 Gel electrophoresis

i) Laemmli (1970) gels.

Table 2.1 outlines the components used in Laemmli gel preparation. The quantities indicated were sufficient for forming one gel of dimensions 140x100x1.5 mm. Acrylamide: bisacrylamide solution, resolving gel buffer and stacking gel buffer were filtered before use, and stored at 4°C, the acrylamide:bisacrylamide being kept in the dark. The

resolving gels were 7.5% acrylamide, 0.2% bisacrylamide, with ammonium persulphate (freshly made) as the polymerization catalyst. Resolving and stacking gel mixtures (without TEMED) were degassed before pouring the gels, the TEMED being added just before pouring. An LKB 2001 vertical electrophoresis unit was used with an LKB 2197 power supply; gels were usually electrophoresed at 35 V overnight or at 200 V for 3-4 hours.

ii) SDS-phosphate continuous gels (Weber and Osborne 1969).

The gels (5% or 7.5% acrylamide) were made as below using the LKB vertical electrophoresis unit. Sodium phosphate buffer was made by preparing 0.5 M solution of Na_2HPO_4 and NaH_2PO_4 and mixing to give the desired pH (approximately 123 ml Na_2HPO_4 and 877 ml NaH_2PO_4).

Acrylamide:bis acrylamide (30:0.8% (w/v))	10 ml
0.5 M sodium phosphate pH 6.0	12 ml
10% (w/v) SDS	0.6 ml
1.5% (w/v) ammonium persulphate	3.0 ml
deionised water	34.4 ml
TEMED	0.03 ml

The reservoir buffer consisted of:

0.5 M sodium phosphate pH 6.0	1000 ml
10% (w/v) SDS	50 ml
distilled water	3950 ml

The gels were electrophoressed at 200 mA for 2 hours, using the same power pack as for the Laemmli gels.

2.3.3 Staining of gels

i) Coomassie Blue staining.

After electrophoresis, gels were transferred to a clear plastic container with an excess of Coomassie stain (0.025% (w/v) Kenacid Blue R in 50% (v/v) methanol and 5% (v/v) acetic acid, filtered before use). The gels incubated with gentle agitation at room temperature for variable lengths of time, until stained bands were visible. If required, the gels were destained in an excess of destain solution (5% (v/v) methanol, 7.5% (v/v) acetic acid) until the bands were clearly visible. The gels were photographed as described below.

On some occasions, the contrast of the Coomassie stained bands was further enhanced by silver staining (Görg *et al.* 1985). All stages were carried out in daylight with gentle agitation. The Coomassie stained gel was washed twice for 5 minutes in 10% (v/v) ethanol, then three times for 3 minutes in distilled water. The gel was incubated for 30 minutes in 0.1% (w/v) $AgNO_3$, and rinsed briefly in distilled water. To develop the silver stain, the gel was immersed in 100 ml of freshly prepared 3% (w/v) $NaCO_3$ containing 0.02% (w/v) formaldehyde. The staining was monitored visually, and the reaction stopped after 5-10 minutes by immersing the gel in 1% (v/v) acetic acid for 10 minutes. Finally the gel was rinsed three times in distilled water and photographed.

ii) Silver staining (Morrisey, 1981).

Following electrophoresis, the gels were transferred to clear plastic containers and incubated in the following solution (100 ml/gel) for 30 minutes. All stages were carried out using solutions made up in ultrapure water. Incubations were at room temperature, in daylight and with agitation.

1. 50% (v/v) methanol, 10% (v/v) acetic acid.
2. 5% (v/v) methanol, 7% (v/v) acetic acid.
3. 50 ml of 10% (v/v) glutaraldehyde.

After these fixation steps, the gel was thoroughly washed with several changes of

deionised water, including an overnight wash. The gels were incubated in 100 ml/gel of 5 μg/ml DTT for 30 minutes, then in 100 ml of 0.1% (w/v) silver nitrate for 30 minutes. To develop the stain, the gels were rinsed once briefly in deionised water, and twice in developer solution (0.05% (v/v) of 37% (v/v) formaldehyde solution in 0.3% (w/v) sodium carbonate). Developer (100 ml) was added, and the reaction monitored visually whilst shaking over a light box. Staining was stopped by adding 5 ml of 2.3 M citric acid for 10 minutes, followed by thorough washing in deionised water. Finally the gel was incubated in 100 ml of 0.03% (w/v) sodium carbonate for 10 minutes and photographed as described below.

2.3.4 Autoradiography

Gels from the phosphorylated intermediate experiments were dried without fixation before autoradiography. This was carried out using a Bio-Rad model 483 slab dryer, set at 80°C for 2 hours. The gels were then exposed to Kodak X-omat S autoradiography film in the presence of a Du Pont Cronex Lighting Plus intensifier screen at -70°C for 1 day (microsome preparations) or 7 days (purified calmodulin-stimulated ATPase).

2.3.5 Photography

Gels and immunoblots were photographed using a Pentax K-1000 camera with macro lens. The film used was Ilford Technical Pan; negatives were produced using Ilford Ilfotec developer and Ilford Hypam fixer. Prints were produced using Ilfospeed paper developer and Ilfotech fixer on Ilfospeed multigrade paper. Gels and autoradiographs were photographed using transmitted light, and blots with reflected light.

2.3.6 Determination of relative molecular mass

For each experiment, lane(s) containing marker proteins were cut from the gel and stained with Coomassie blue stain as above. The R_f value of the marker proteins was

calculated and plotted against log M_r. Representative calibration curves for Laemmli and Weber and Osborne gels are shown in Figures 2.3 and 2.4 respectively. A linear regression line was fitted to the points and used in calculation of M_r values of sample proteins.

2.4 METHODS: Use of monoclonal antibodies

Production of the monoclonal antibodies is described in Appendix III.

2.4.1 Immunoblotting

Samples of washed microsomal proteins or calmodulin-stimulated ATPase were electrophoresed on 7.5% neutral SDS polyacrylamide gels as described in sections 2:3:1 and 2:3:2. Samples of erythrocyte ghost protein were supplied by P. James (Eidgenössiche Technische Höchschule, Zürich) in lyophilised form, and were taken up in SDS sample buffer (with PMSF and chymostatin) and loaded onto a 7.5% SDS polyacrylamide minigel as described previously.

After electrophoresis, lanes with molecular weight markers and with enzyme or membrane samples were removed and stained for protein using Coomassie blue dye or silver stain as described in section 2:3:3.

For immunoblotting, proteins from the SDS gels were electrophoretically transferred to nitrocellulose membranes by the method of Towbin *et al.* (1979). The transfer membrane used was Schleicher and Schuell, 0.2 μm pore size, grade BA 83. Transfer buffer was 25 mM Tris-HCl, pH 8.3, 192 mM glycine, 20% (v/v) methanol, and transfer was carried out at 50 V for 3 hours. Nitrocellulose membranes to be stained for protein content were immersed in an excess of 'AuroDye forte' (Janssen, ICN, High Wycombe, Bucks., U.K.) for 10-20 minutes at room temperature and with gentle agitation. The membranes to be used for immunoblotting with the monoclonal antibodies produced in this study were blocked for 3 hours with 5% (w/v) BSA (immunoglobulin-free) in

Tris-buffered saline (TBS, 0.02 M Tris-HCl, pH 8.2, 0.15 M NaCl, 0.01 M sodium azide) at room temperature with constant agitation. When the monospecific polyclonal antibody 97.1 was used, the membranes with transferred protein were blocked in 10% (w/v) milk powder ('Easypints'; J. Sainsbury, London) in phosphate-buffered saline with azide (PBSA, 0.2 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.02% (w/v) sodium azide). Subsequent additions to immunoblots with the monoclonal antibodies were made in 0.1% (w/v) BSA in TBS (BSA-TBS); to immunoblots with polyclonal antibody 97.1, additions were made in 0.5% (w/v) milk powder in PBSA (milk-PBSA). Between each stage membranes were washed three times in BSA-TBS or milk-PBSA as appropriate for 5 minutes. The transferred proteins were incubated with gentle agitation overnight in the following solutions:

1) Undiluted cell free hybridoma supernatants, with the addition of 1% (v/v) normal goat serum.

2) Polyclonal serum diluted 800-fold.

3) Polyclonal antibody 97.1 (produced by F. Kessler, Eidgenössiche Technische Höchschule, Zürich) diluted 10-fold.

4) Control strips were incubated as appropriate in milk-PBSA, BSA-TBS, or tissue culture medium (TCM, RPMI 1640 with appropriate amount of foetal bovine serum). Controls were also incorporated, where applicable, with antibodies that were raised to antigens other than the CSCPA.

Binding of the antibodies was visualised using Janssen blotting grade AuroProbe polyvalent goat anti-mouse (for monoclonals) or anti-rabbit (for antibody 97.1) gold conjugate. The gold-conjugated antibody was diluted 200-fold in BSA-TBS with 5% (v/v) liquid gelatin (supplied with the gold-conjugate). Filters were incubated for 24-48 hours. The gold signal was enhanced with a Janssen silver enhancement kit used according to the manufacturers' instructions

2.4.2 Effect of monoclonal antibodies on calmodulin-stimulated ATPase activity in microsomes

The effect of the monoclonal antibodies on the calmodulin stimulated component of ATPase activity in washed microsomes was assayed by inclusion into the radioisotope assay. Cell-free supernatants were diluted to give a range of concentrations in TBS. The diluted supernatants were included in the assay mixture, and the assay carried out as described previously. The effect of the antibodies on calmodulin stimulated enzyme activity relative to that of controls was assessed, after correction had been made for the stimulatory effect of TBS.

Figure 2.1: Representative calibration curve for spectrophotometric ATPase assay.

Values are means (n = 3)

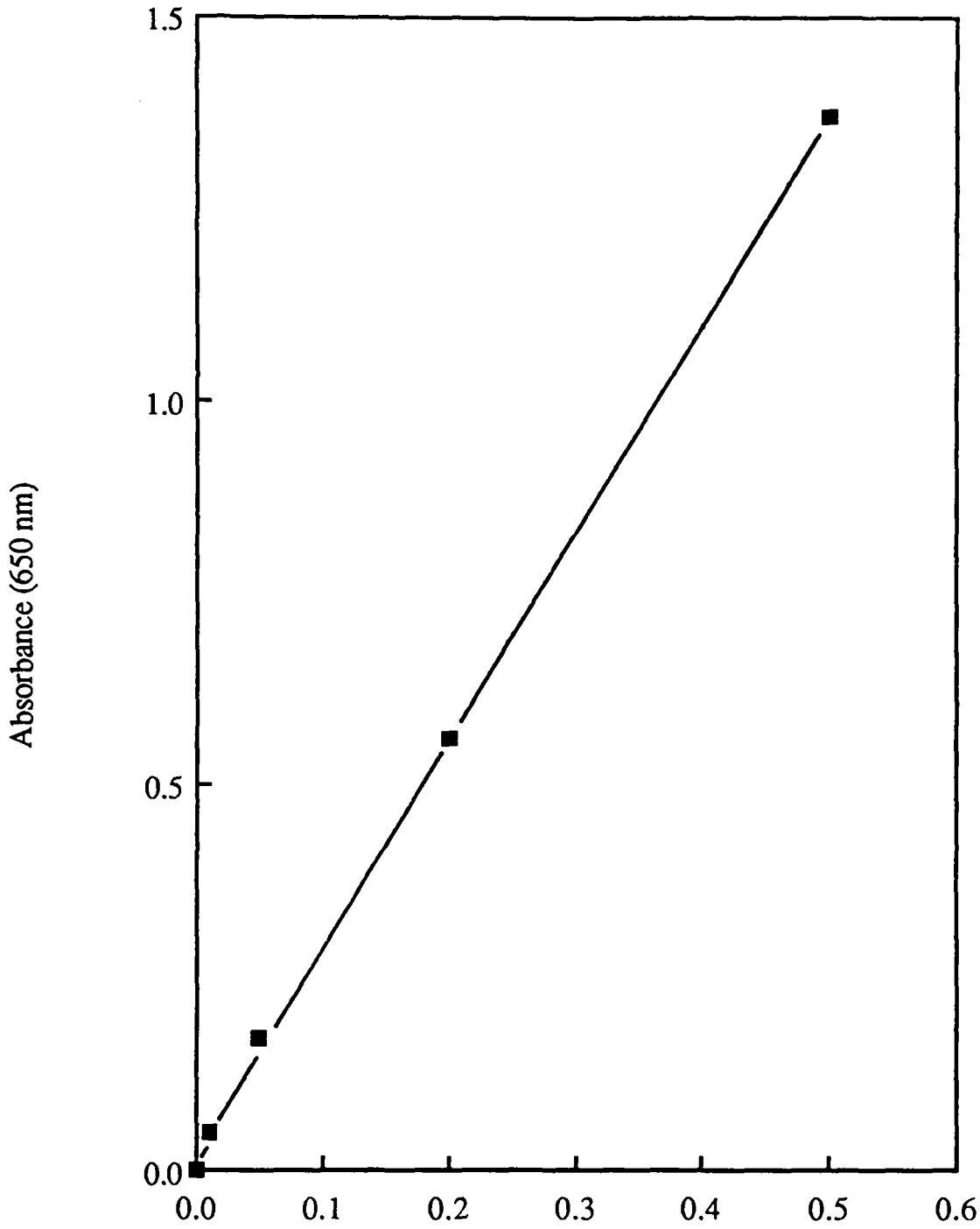
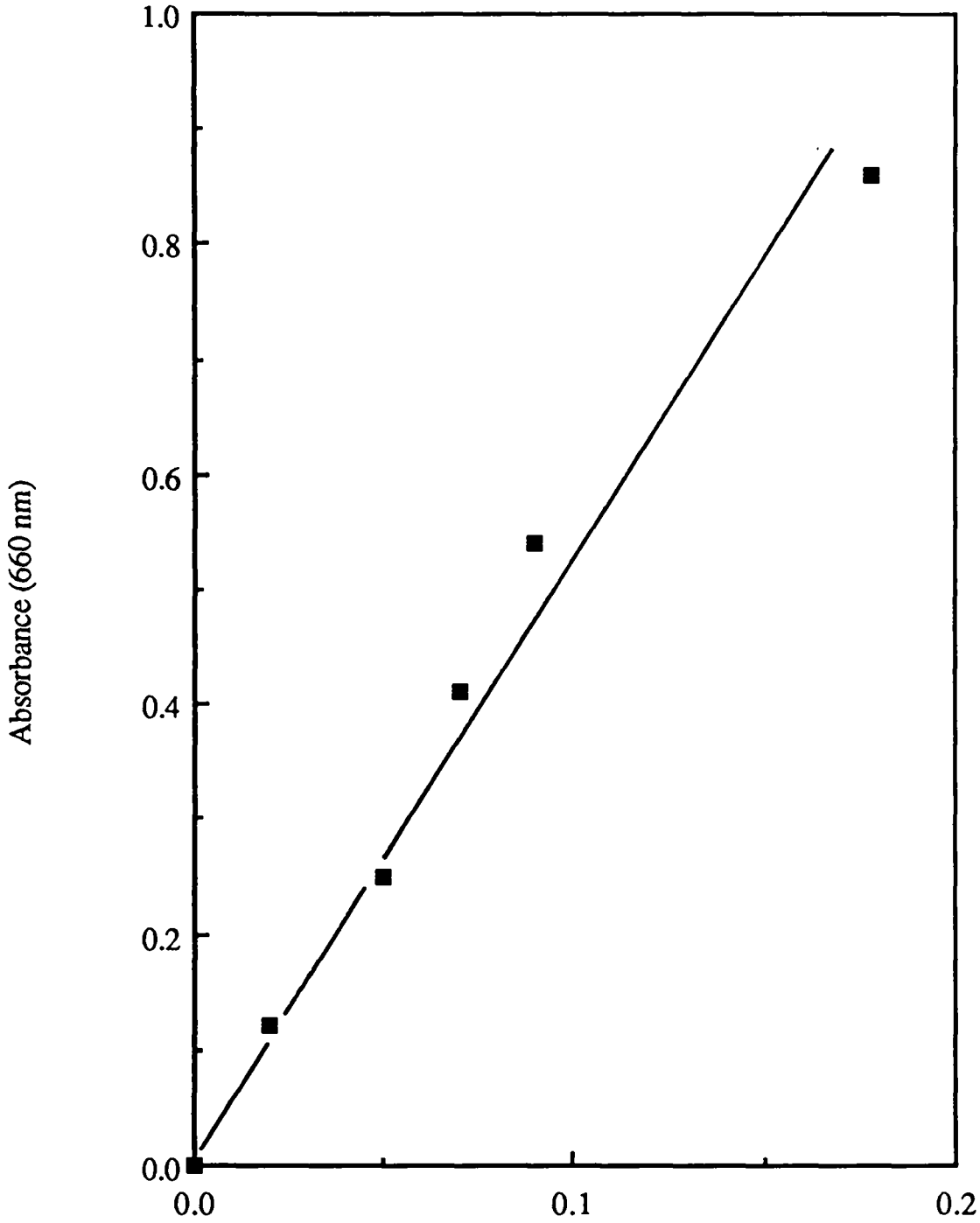

Absorbance (650 nm)
1.5
1.0
0.5
0.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
µmoles Pi per assay

Figure 2.2: Representative calibration curve for protein assay of Bensadoun and Weinstein (1976).

Values are means of triplicate determinations.


1.0
0.8
0.6
0.4
0.2
0.0
Absorbance (660 nm)
0.0
0.1
0.2
Amount protein per assay (mg)

Stock solution	Stacking gel (ml)	Resolving gel (ml) final acrylamide concentration		Reservoir buffer (ml)
		5%	7.5%	
Acrylamide: bisacrylamide (30:0.8)	2.5	5.0	7.5	--
Stacking gel buffer stock[a]	5.0	--	--	--
Resolving gel buffer stock[b]	--	3.75	3.75	--
Reservoir buffer stock[c]	--	--	--	500
10% SDS	0.2	0.3	0.3	--
1.5% ammonium persulphate	1.0	1.5	1.5	--
Water	11.3	19.45	16.95	4500
TEMED	0.015	0.015	0.015	--

Table 2.1: Components used in preparation of neutral SDS-polyacrylamide gels.

[a] Stacking gel buffer stock: 0.5 M Tris-HCl (pH 6.8)

[b] Resolving gel buffer stock: 3.0 M Tris-HCl (pH 8.8)

[c] Reservoir buffer stock: 0.25 M Tris, 1.92 M glycine, 1% SDS (pH 8.3)

Figure 2.3: Representative calibration curve for relative molecular mass markers on Laemmli SDS (7.5%) polyacrylamide gel.

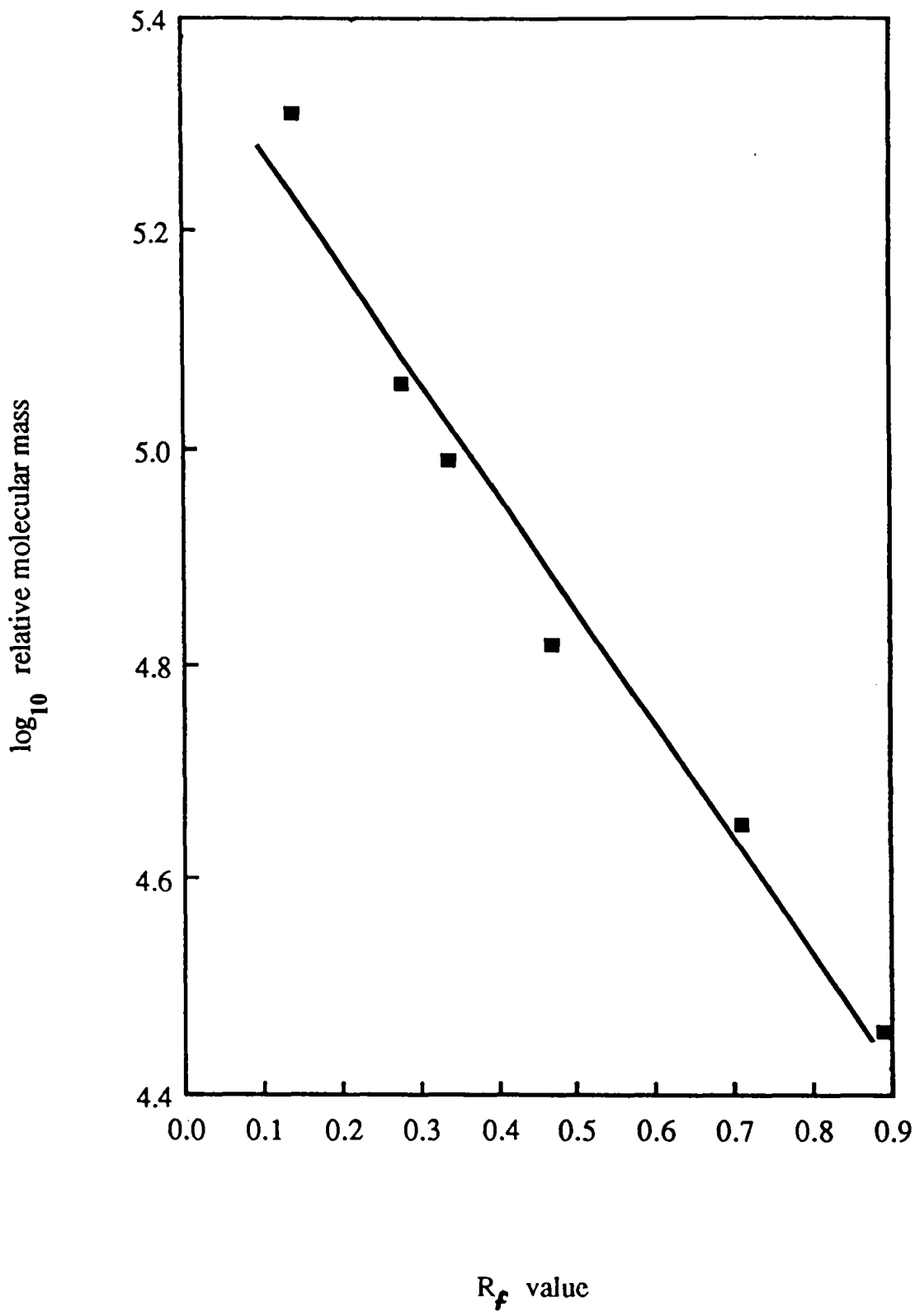
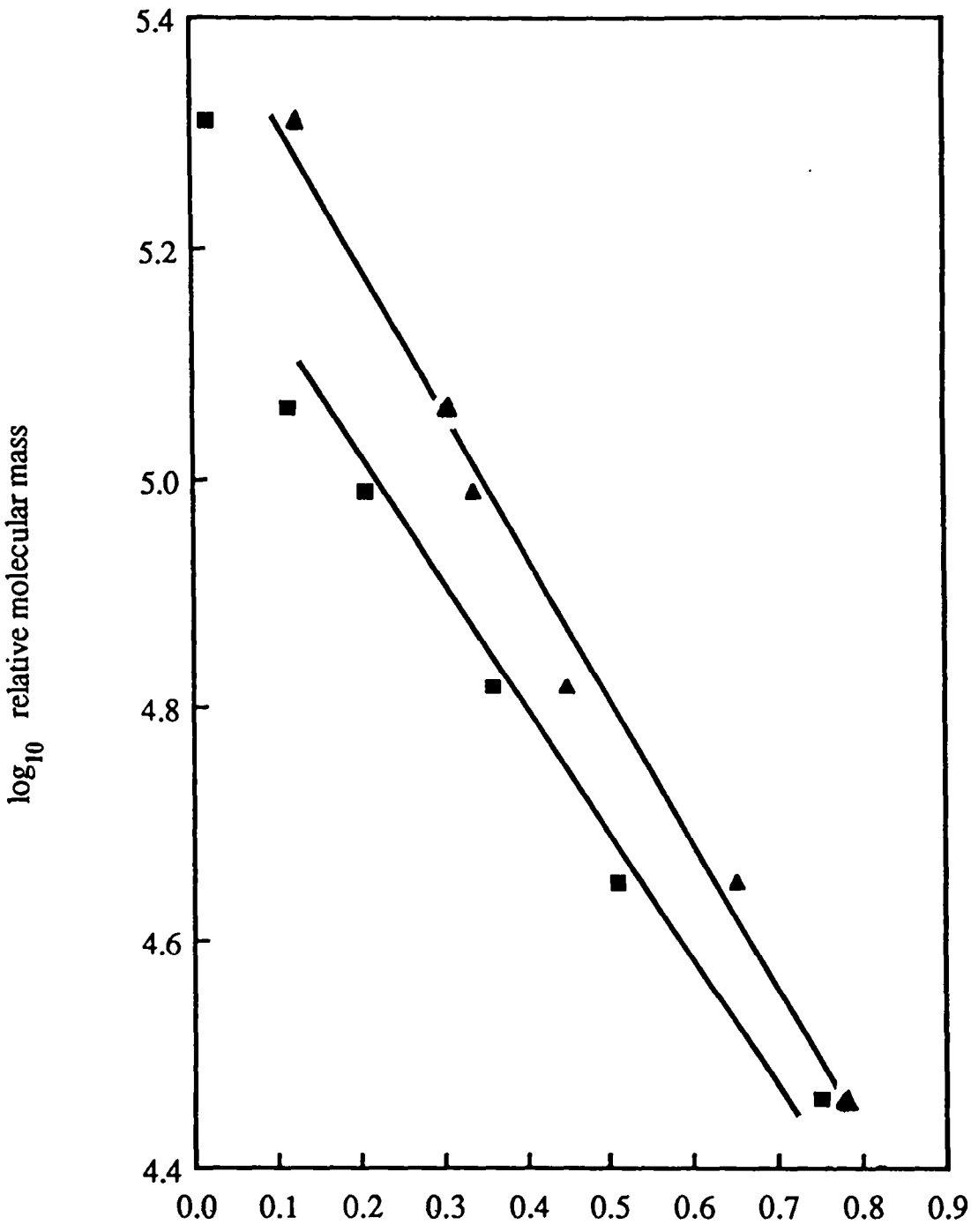

5.4
5.2
5.0
4.8
4.6
4.4
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
log10 relative molecular mass
Rf value

Figure 2.4: Representative calibration curve for relative molecular mass markers on Weber and Osborne 7.5% (■) and 5% (▲) SDS polyacrylamide gel.


5.4
5.2
5.0
4.8
4.6
4.4
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
log10 relative molecular mass
Rf value

Chapter 3

STUDIES ON CALMODULIN-STIMULATED ACTIVITY IN MAIZE MICROSOMES

3.1 INTRODUCTION

3.1.1 Background

As indicated in Chapter 1, evidence accumulated concerning the properties of calcium transport in microsomal or purified plasma membrane preparations strongly indicates that a calcium-pumping ATPase is present in plant tissues, which is located at the plasma membrane, and may be stimulated by calmodulin. Unequivocal proof of the presence of this calcium transporter required further purification and characterisation, an objective which was the aim of the study presented in this thesis. At the onset of this study, the only reported purification of a plant calmodulin-stimulated ATPase was that of Dieter and Marmé (1981b) in which a microsome fraction from dark grown maize

coleoptiles was used as the starting material. It was decided that success in purification of a calcium pumping ATPase was most likely to be achieved using a membrane extract which had been shown to exhibit calmodulin-stimulation of ATPase and calcium transport activities, since this permitted a relatively simple purification protocol to be used. Before purification of the calmodulin-stimulated, calcium pumping ATPase (CSCPA) was commenced it was necessary to develop sensitive assays for detection of ATPase activity, and to confirm that calmodulin-stimulated ATP hydrolytic activity and calmodulin-stimulated calcium transport were present in the tissue to be extracted. This chapter describes development of assays for ATPase activity and demonstration of the presence of calmodulin-stimulated ATPase activity in microsomes extracted from dark grown coleoptiles of *Zea mays* L. cv. Golden Bantam. Appendix I contains supplementary information regarding the demonstration of calmodulin-stimulated calcium uptake in a microsomal preparation.

3.1.2 Methodology

Methods for the production of microsomes were based on Dieter and Marmé (1981b). During the course of this study, it was found to be necessary to modify these in order to produce reliable and reproducible assays. Details of the protocols used have been described (Methods 2:2:1, 2:2:2, 2:2:4).

i. Production of microsomal vesicles.

Zea mays L. c.v. Golden Bantam was used in the work described here since stock which had not been mercury treated was available. Dark grown seedlings were used for two reasons. Firstly, the majority of studies published before this work had demonstrated calmodulin stimulation of calcium transport and ATPase activities only in etiolated tissues. Secondly, the absence of choloroplasts reduced contamination during cell fractionation.

In the preparation of membrane fractions from plant cells, it is important to work

rapidly, since disruption of the tonoplast membrane releases a variety of materials from the vacuole, which frequently have deleterious effects on subsequent purification. Homogenisation of coleoptiles was carried out by hand chopping followed by grinding without abrasives in a mortar. This was sufficient to break the cell walls but not to disrupt membrane-bound organelles. The medium used provided a suitable osmoticum (10% (w/v) sucrose) together with a buffer system which prevented changes in pH that may occur on release of vacuolar contents. The combination of sucrose and MOPS/Tris is suitable for preparation of fractions containing plasma membranes, being similar to the osmoticum/buffer systems described by Hodges and Leonard (1974) and Hall (1983). Addition of EDTA to the homogenisation medium chelated endogenous divalent cations and resulted in removal of calmodulin from the membranes. EDTA may provide some protection against protease activity since it functions as a metalloprotease inhibitor (Evans, 1987). In addition, chelation of heavy metals by EDTA probably reduces phospholipase activity, so preserving membrane structure (Hall, 1983). Filtration of the homogenate through muslin layers removed cell wall debris which would interfere with subsequent centrifugation.

The use of differential centrifugation to separate membrane components is based on the sedimentation and density properties of different organelles. Centrifugation of the supernatant at 48,000xg for 20 minutes produced a mixed membrane pellet of microsomal origin, suggested by Gross and Marmé (1978) to contain plasma membrane. In early studies, centrifugation at 48,000xg was carried out once, producing 'crude' microsomes. Resuspension of this pellet, followed by a second period of centrifugation at 48,000xg, yielded a 'washed' microsomal fraction. The purpose of this additional centrifugation was to remove loosely bound extrinsic membrane proteins, especially non-specific phosphatases, which would interfere with subsequent assays. In later experiments, additional protease inhibitors were included. Dithiothreitol (DDT) reduced -S-S- groups to -SH

groups and hence functions as a sulphydryl protector. Hodges and Leonard (1974) reported that inclusion of DDT in extraction and final suspension buffers was necessary in order to maintain ATPase activity over extended time periods. Phenylmethylsulphonyl fluoride (PMSF) was included because it specifically inhibits serine proteases. It has a half-life of 100 minutes in aqueous solutions and was therefore added fresh to all buffers and replenished if necessary.

ii. ATPase assays.

a) Measurement of ATPase activity.

Crude or washed microsomes were resuspended in a buffer containing 0.02% (w/v) Triton X-100. The function of this was to permeabilise the membranes, such that more ATP hydrolytic domains were exposed. BSA was included in the incubation medium as a means of reducing the effects of proteases on the calmodulin-stimulated ATPase. Oligomycin, a known inhibitor of mitochondrial calcium uptake, was also included in the incubation medium.

The principle of the spectrophotometric assay for release of inorganic phosphate (Pi) (Scherer and Morré, 1978) lies in the addition of an ascorbate/molybdate/sulphuric acid mixture to the reaction medium after removal of TCA-precipitated proteins. This mixture complexes inorganic phosphate released during the reaction, producing a coloured compound, the absorbance of which may be determined spectrophotometrically. This method measured phosphate released from any source, not just from ATP hydrolysis. Suitable controls were included in all experiments so that Pi released in the absence of ATP could be subtracted from that in the presence of ATP. Similar spectrophotometric assays for release of inorganic phosphate have been described in other reports of calmodulin-stimulated ATPase activity (Dieter and Marmé, 1981b; Robinson *et al.* , 1988).

In order to characterise calmodulin-stimulation of microsomal ATPase activity further, a second assay system was developed. The approach chosen was based on the

report of Seals *et al.* (1978). This method measures the release of ^{32}P from [$\gamma^{32}P$]-ATP, and hence only phosphate liberated by ATP hydrolysis. In this assay, SDS was used to terminate the reaction, and phosphomolybdate formation was initiated by the addition of a sulphuric acid/ammonium molybdate/silicotungstic acid mixture. A xylene/isobutanol mixture was used to extract the phosphomolybdate complex, separating ^{32}P from [$\gamma^{32}P$]-ATP, and this was quantified by scintillation counting. This assay procedure was reported to be rapid, minimise acid-induced ATP hydrolysis, and to be capable of extracting 10^{-7} to 10^{-15} moles Pi (Seals *et al.* , 1978). The sensitivity of this assay system made it suitable for use in the measurement of ATPase activity of purified CSCPA. In addition use of this assay for measurement of ATPase activity by purified CSCPA gave more reproducible results than the spectrophotometric assay.

b) Nucleotide triphosphate specificity

Studies of calcium transport by higher plant cell membranes have shown generally low nucleotide specificity (ATP 100% , GTP 50% , ITP 40% , mixed plasma membrane/Golgi fraction, pea root, Butcher and Evans, 1987a; ATP 100% , GTP 37% , UTP 27% , CTP 17% , basal activity from maize, Gross and Marmé, 1978; ATP 100% , GTP 25% , ITP 30% , CTP 5% , ER, cress roots, Buckhout, 1983; ATP 100% , GTP 50% , red beet plasma membrane, Giannini *et al.* , 1987a). However, the nucleotide specificity of the mammalian plasma membrane calcium transporter is very high, activity being negligible other than with ATP (Rega *et al.* , 1973) and it was therefore important to determine the nucleotide specificity of the higher plant CSCPA.

c) Use of sodium orthovanadate as an inhibitor of ATPase activity.

Orthovanadate appears to inhibit plasma membrane-type (but not tonoplast-type) cation transporting ATPases (I_{50} 5 μM, subject to experimental conditions). The inhibitory effect of orthovanadate on red blood cell CSCPA was reported by Barrabin *et al.* (1980) and Bond and Hudgins (1980). Orthovanadate has also been suggested to be an inhibitor of plant H^+-ATPase (Gallagher and Leonard, 1982); however Dieter and

Marmé (1980c) did not find $2x10^{-5}$ M orthovanadate to be an inhibitor of microsomal calcium transport in maize, whilst Zocchi and Hanson (1983) showed 30% inhibition at 0.1 mM orthovanadate. It was therefore important to establish the real effect of vanadate on the higher plant CSCPA.

3.2 RESULTS

3.2.1 Spectrophotometric ATPase assay

i) Development of assay.

Attempts were made to reproduce the method of Dieter and Marmé (1981b) for measurement of calmodulin-stimulated ATPase activity in microsomes from 5.5 day old maize coleoptiles. The experiments carried out were not successful (data not shown) mainly because of high variation in the basal (without calmodulin) values. It was found that four modifications to the assay system of Dieter and Marmé were required in order to produce a reliable assay. These were as follows:

1) Extraction of microsomes from 4.5 day old coleoptiles:

2) Resuspension of the first 48,000xg pellet in Basic Buffer containing 1 mM DTT:

3) Washing the microsome pellet:

4) Resuspending the washed (second) microsome pellet in Buffer B2 containing Triton X-100 and including Triton X-100 in the incubation medium.

These modifications have been described in full in Chapter 2 (section 2:2:2). These modifications permitted development of a reliable assay in which it was possible to demonstrate calmodulin-stimulation of ATPase activity over a four hour time period (Figure 3.1). In the presence of 0.275 μM calmodulin, stimulation of ATPase activity was 4.2 nmoles min^{-1} (mg protein)$^{-1}$ 30 minutes after washed microsome production, 3.8 nmoles min^{-1} (mg protein)$^{-1}$ after two hours, 3.9 nmoles min^{-1} (mg protein)$^{-1}$ after three hours and 4.6 nmoles min^{-1} (mg protein)$^{-1}$ after four hours. It can be seen from Figure 3.1 that over this time period basal ATPase activity declined steadily from a value of 58.8 nmoles min^{-1} (mg protein)$^{-1}$ 30 minutes after production of washed microsomes to 47.1 nmoles min^{-1} (mg protein)$^{-1}$ four hours after production of microsomes. This assay method was considered to be sufficiently reliable, and was used in all subsequent studies of ATPase activity using the spectrophotometric assay.

ii) Nucleotide triphosphate specificity of calmodulin-stimulation of inorganic phosphate release in microsomes from 4.5 day old coleoptiles.

As can be seen in Figure 3.2, the highest nucleotide triphosphate-dependent inorganic phosphate release occurred in the presence of 1 mM ATP. Taking the value for inorganic phosphate release in the presence of 1 mM ATP (in the absence of calmodulin) as 100% , the percentage activities (without calmodulin) in the presence of 1 mM of other nucleotide triphosphates were as follows: GTP 72% , ITP 61% ,UTP 65% . Calmodulin-stimulated inorganic phosphate release was maximal with 1 mM ATP as substrate (100%); with GTP it was 6% , with ITP 33% and with UTP 18% .

iii) Effect of sodium orthovanadate.

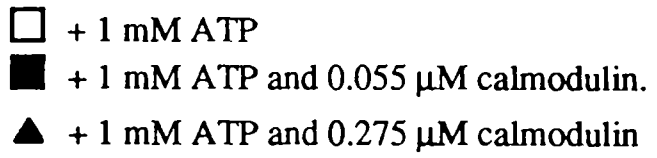
Washed microsomes were incubated in the presence or absence of saturating (0.3 μM) calmodulin and with various concentrations of orthovanadate. The results in Figure 3.3 indicate that basal ATPase activity was inhibited at all concentrations of orthovanadate, with an I_{50} of 5 μM. Calmodulin-stimulated component of ATPase activity was inhibited by orthovanadate, with an I_{50} of about 20 μM.

3.2.2 Radioisotope ATPase assay

The radioisotope assay was set up as described (Methods 2:2:4) using the same buffers as the spectrophotometric assay (i.e. DDT and Triton X-100 in Buffer B2, BSA and Triton X-100 in incubation medium). In Figure 3.4 data derived from the two assay methods for calmodulin-stimulation of ATPase activity in washed microsomes is compared. These two data sets were from representative experiments, repeated four times; further comment on this may be found in section 3:3. For the radioisotope assay, the basal ATPase activity was 25.1 nmoles min^{-1} (mg protein)$^{-1}$, as compared with 51.5 nmoles min^{-1} (mg protein)$^{-1}$ for the spectrophotometric assay. Over the range of calmodulin concentrations used, the maximum stimulation values were 7.8 nmoles min^{-1} (mg protein)$^{-1}$ in the spectrophotometric assay and 8.0 nmoles min (mg protein)$^{-1}$ in the radioisotope

assay. The assays produced different results in terms of the calmodulin concentration at which half-maximal stimulation was achieved. For the range of calmodulin concentrations used, it appeared that maximal stimulation was not reached by 0.35 μM in the spectrophotometric assay. The dose-response curve for the radioisotope assay was not as smooth as for the spectrophotometric assay, maximal stimulation being achieved between 0.05 and 0.11 μM calmodulin, and therefore half maximal stimulation was at 0.025-0.05 μM. It appeared that at concentrations of calmodulin greater than 0.11 μM the extent of calmodulin-stimulation decreased.

Figure 3.1: Time course of calmodulin stimulation of ATPase activity in washed microsomes from 4.5 day old maize coleoptiles, spectrophotometric assay.



Representative of two experiments; points are means plus or minus one standard deviation (n =2)

Coleoptiles of 4.5 day old dark grown maize seedlings were homogenised in 2 ml: 1 g Buffer A (10% sucrose, 25 mM MOPS, brought to pH 7.5 with solid Tris and 5 mM EGTA) and centrifuged at 6,000xg (15 minutes). Microsomes were prepared by two centrifugations at 48,000xg for 20 minutes, the final pellet was resuspended in Buffer B2 (as Buffer A, but with out EGTA and with 1 mM DTT and 0.02% Triton X-100). Assays of calmodulin-stimulated ATPase activity were carried out in incubation medium (as Buffer A, without EGTA, but with 5 mM $MgCl_2$, 1 mM $CaCl_2$ 5 mg/ml BSA, $1x10^{-6}$ M oligomycin and 0.02% Triton X-100. To this, ATP (1 mM) and calmodulin (0-0.275 µM) were added as appropriate. The reaction was initiated by addition of microsome preparation and inorganic phosphate released was detected after termination with TCA by formation of a phosphomolybdate complex as described by Scherer and Morre (1978). Control assays without ATP were carried out and subtracted from the samples containing microsomes. The amount of phosphate released was determined by comparison with a calibration curve constructed for each assay (see Figure 2.1).

This method was followed in Figures 3.2, 3.3, and part of 3.4, with the additions noted in these legends.

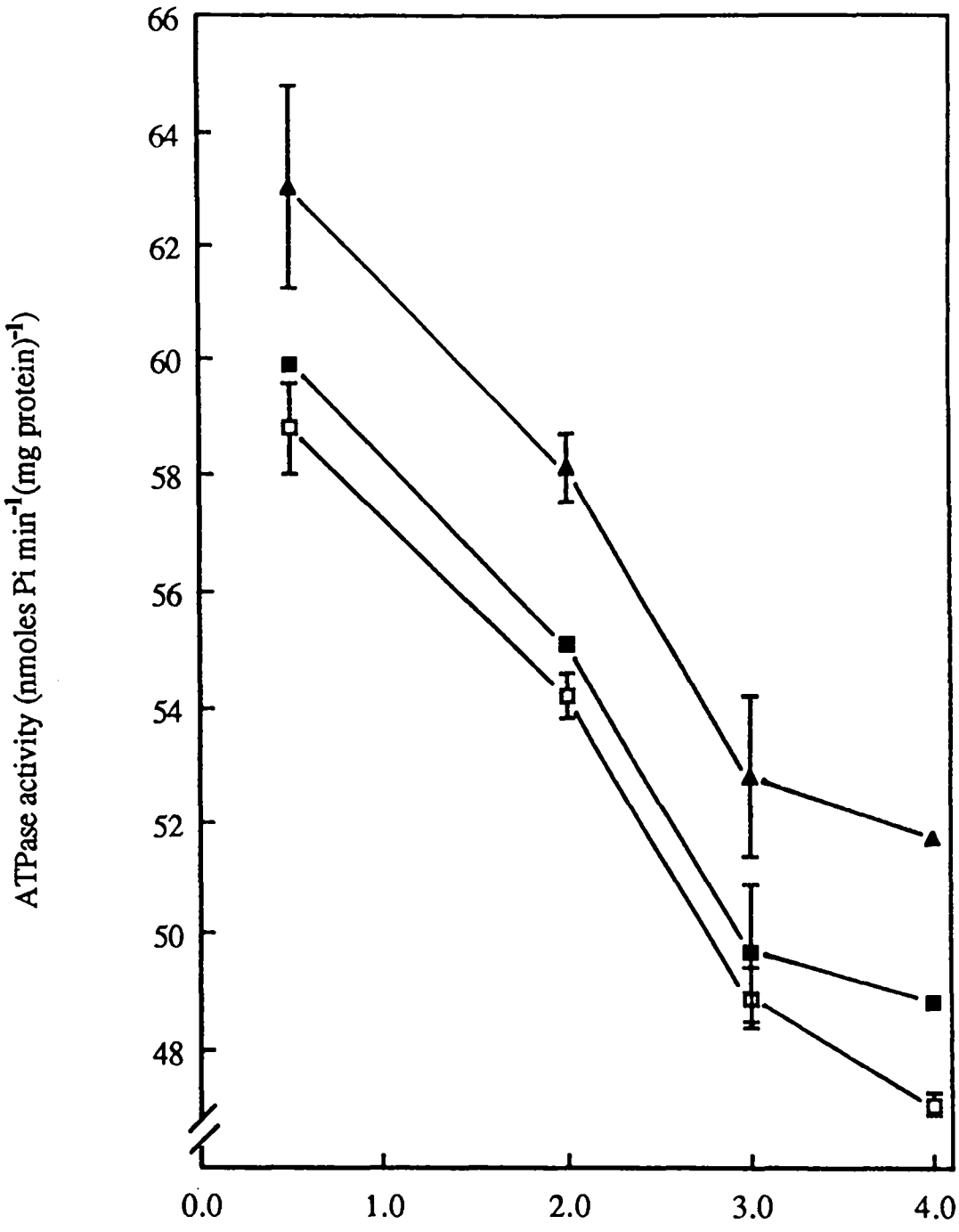

ATPase activity (nmoles Pi min-1(mg protein)-1)
66
64
62
60
58
56
54
52
50
48
0.0
1.0
2.0
3.0
4.0
Time after resuspension (hours)

Figure 3.2: Effect of nucleotides on calmodulin-stimulated Pi release from washed microsomes of 4.5 day old coleoptiles: spectrophotometric assay.

■ + 1 mM ATP

□ +1 mM GTP

◆ + 1mM UTP

▲ +1 mM ITP

Representative of two experiments; values are means plus or minus one standard deviation (n = 3)

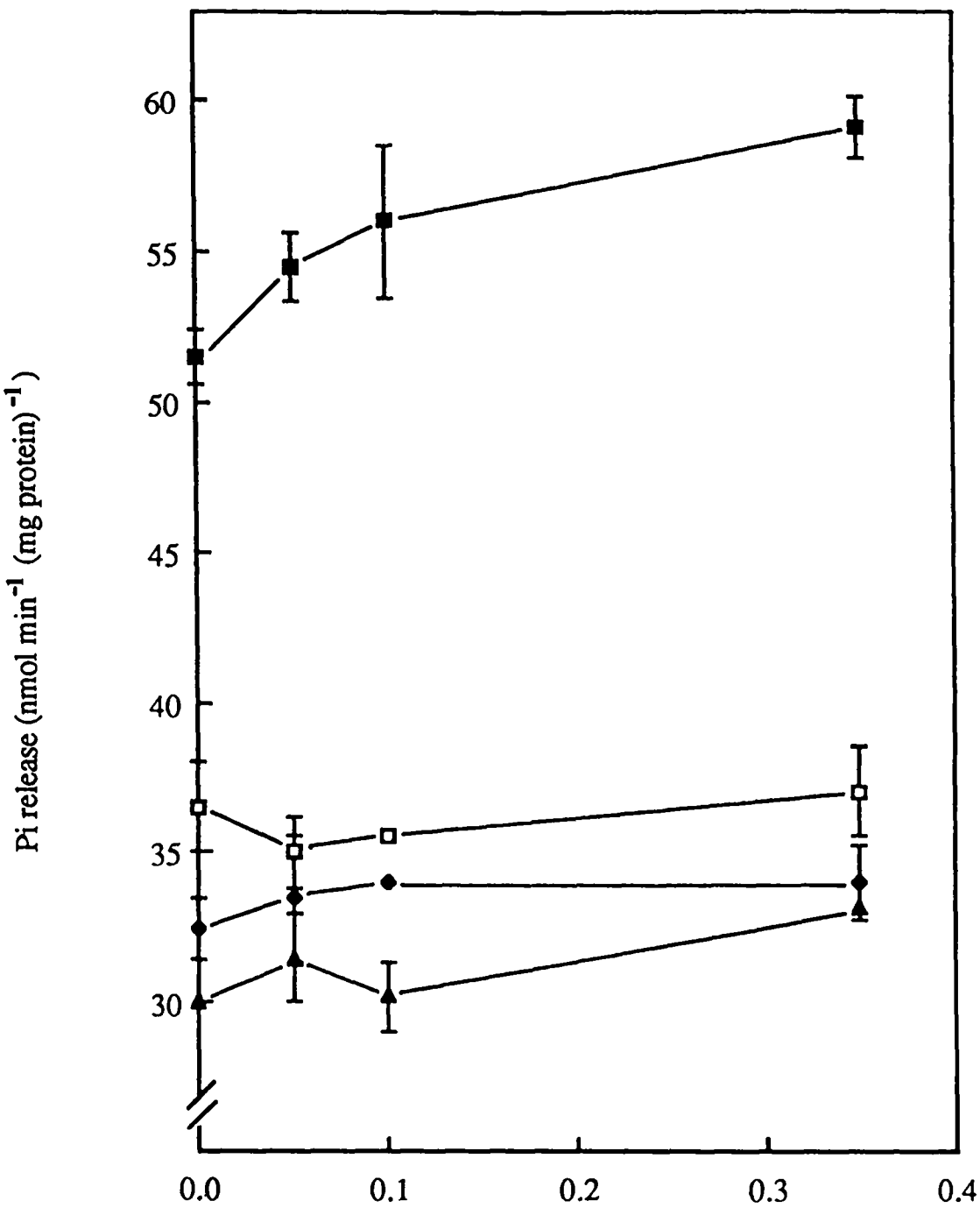

Pi release (nmol min-1 (mg protein)-1)
60
55
50
45
40
35
30
0.0
0.1
0.2
0.3
0.4
[Calmodulin] (μM)

Figure 3.3: Effect of sodium orthovanadate on calmodulin-stimulated and basal ATPase activity in washed microsomes; spectrophotometric assay.

■ + 1 mM ATP and 300 μM calmodulin

□ + 1 mM ATP

◆ Inhibition of calmodulin-stimulated component

Representative of two experiments; values are means plus or minus one standard deviation (n = 3)

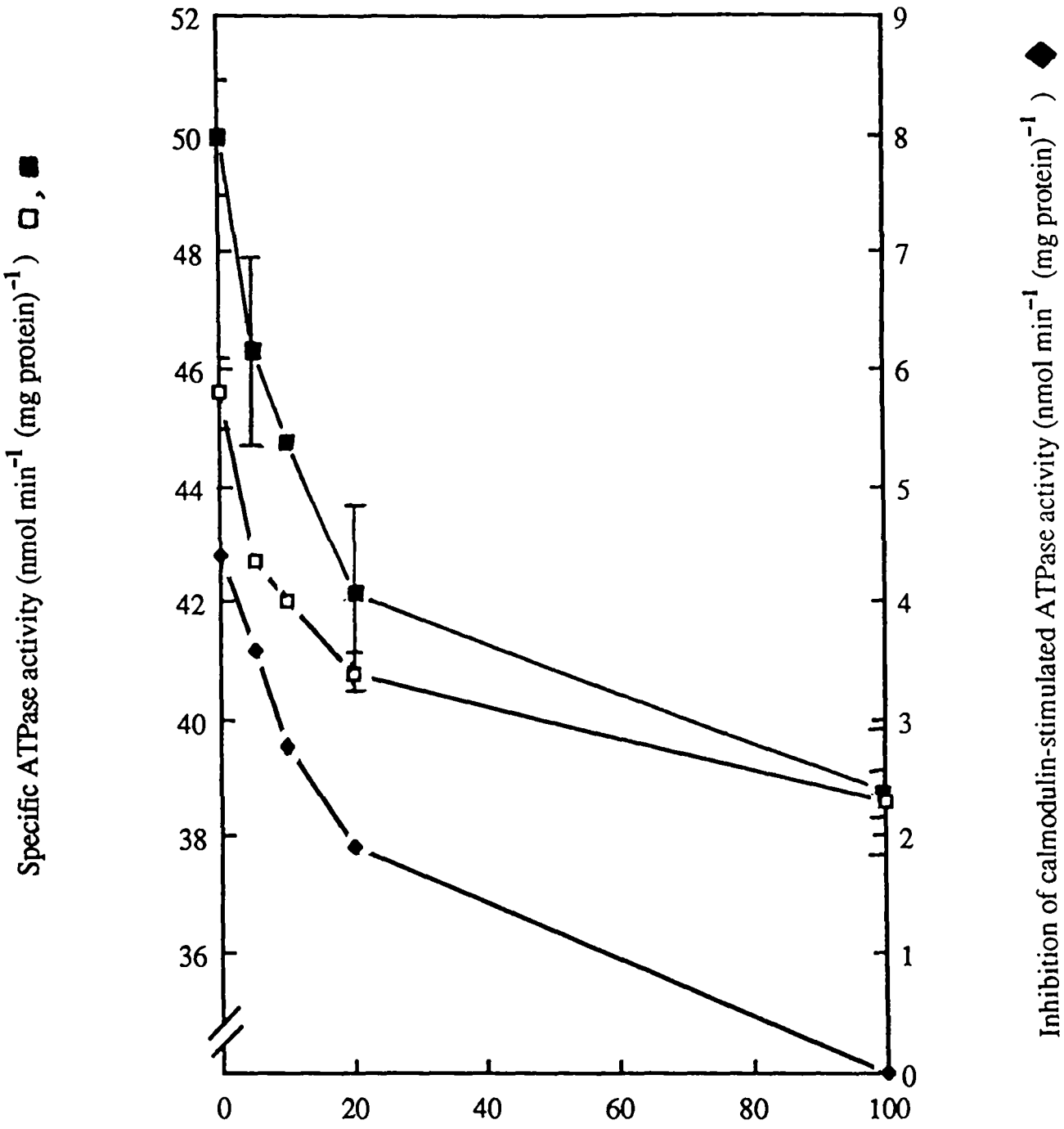

Specific ATPase activity (nmol min-1 (mg protein)-1) □, ■
Inhibition of calmodulin-stimulated ATPase activity (nmol min-1 (mg protein)-1) ◆
52
50
48
46
44
42
40
38
36
9
8
7
6
5
4
3
2
1
0
0
20
40
60
80
100
[Vanadate] (μM)

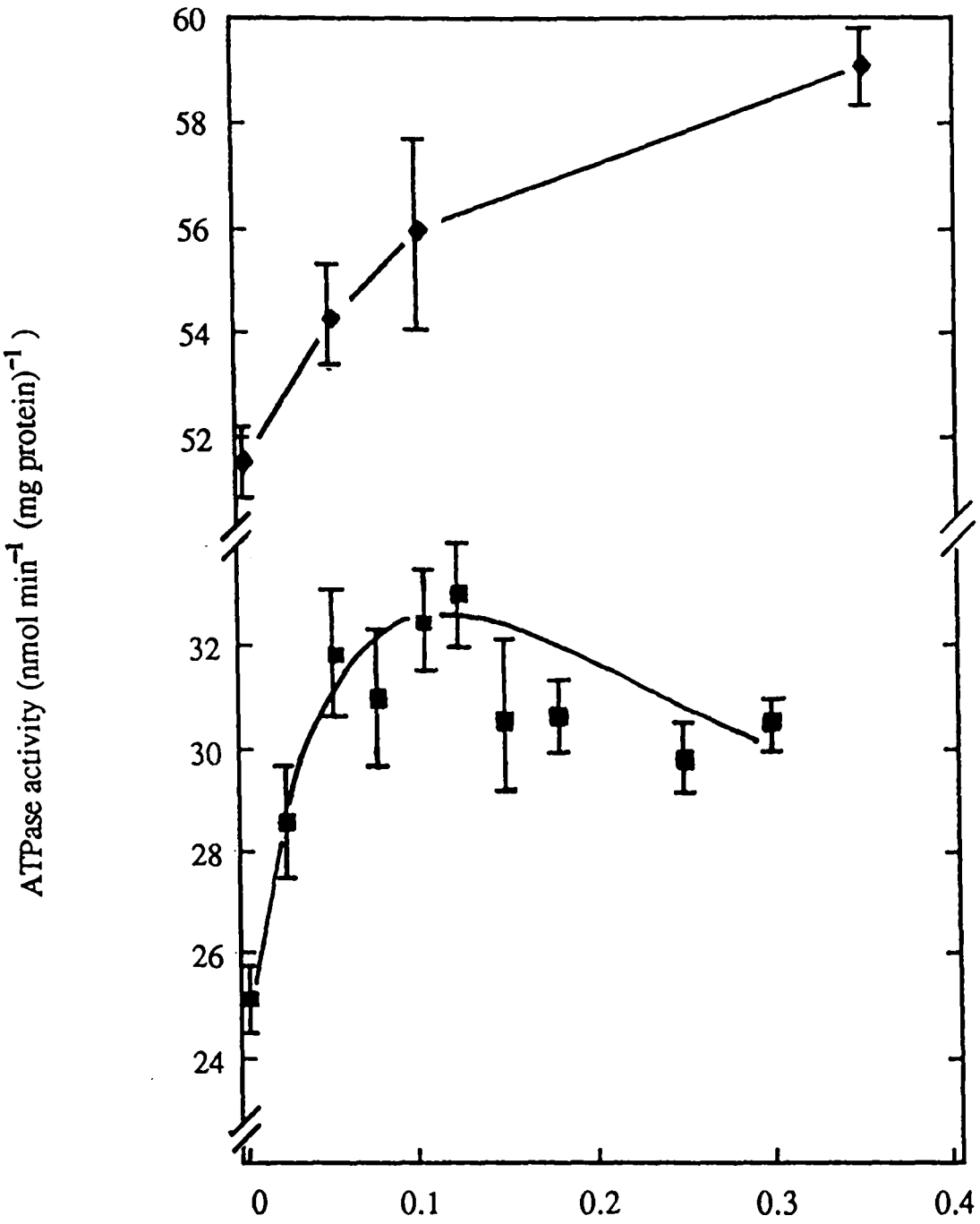
Figure 3.4: Comparison of effect of calmodulin on ATPase activity in washed microsomes as determined by spectrophotometric (◆) assay and radioisotope (■) assay.

Representative of four experiments for Pi release, two experiments for ^{32}P release; values are means plus or minus one standard deviation (n = 3).

Spectrophotometric assay and preparation of microsomes as described in Figure 3.1.

For determination of ATPase activity by release of ^{32}P from [γ^{32} P]-ATP (Seals *et al.*, 1978) assays were set up using the same components as for the spectrophotometric assay, with the addition that 0.1-1.0 μCi [γ^{32} P]-ATP was added to each assay. Before addition of microsomes (or purified calmodulin-stimulated ATPase) a sample of the assay mixture was taken for quantification of the total amount of radioactivity added. Microsomes were added and the samples incubated at 37°C for 30 minutes. The reaction was terminated with 1% SDS. [γ^{32}P] was extracted by addition of phosphate reagent (ammonium molybdate/silicotungstic acid/sulphuric acid) followed by a xylene/isobutanol mixture. After separation of the aqueous and organic phases, a sample of organic phase was taken and the amount of ^{32}P determined.

This method was also used in Figures 4.3, 4.4, 4.8, 4.10, and 6.5.


60
58
56
54
52
32
30
28
26
24
0
0.1
0.2
0.3
0.4
ATPase activity (nmol min^{-1} (mg protein)$^{-1}$)
[Calmodulin] (μM)

3.3 DISCUSSION

Results presented in this chapter confirm the presence of calmodulin-stimulated ATP hydrolytic activity in microsomes from maize coleoptiles. This was achieved by modification of assay procedures described by Dieter and Marmé (1981b) for ATPase activity. Data presented in Appendix I shows that the microsome fraction also showed calmodulin-stimulated calcium transport activity.

3.3.1 Spectrophotometric ATPase assay

i) Modification of the ATPase assay of Dieter and Marmé (1981b).

Attempts were initially made to reproduce the methods and results of Dieter and Marmé (1981b) for calmodulin-stimulation of ATPase activity. In order to produce an assay which reliably demonstrated calmodulin-stimulated ATPase activity, the assay procedure was modified in four ways from that described by Dieter and Marmé (1981b), as indicated in section 3:2.

The amount of calmodulin required for maximal stimulation of microsome Pi release was between 0.1 and 0.3 μM in the spectrophotometric assay (Figure 3.4). This compares favourably with Dieter and Marmé (1981b) who showed stimulation by 0.1 μM calmodulin which increased rapidly to maximal by 0.3 μM. Robinson *et al.* (1988) presented a calmodulin dose response curve for ATPase activity which showed maximum stimulation at 1 μM added calmodulin, stimulation at 0.1 μM calmodulin being about 50% , and at 0.3 μM about 70% . The most likely explanation for differing dose response curves is in the experimental conditions used and in variation of the activity of calmodulin preparations. The important point established in this chapter was that calmodulin-stimulated ATPase activity was present. It has also been demonstrated (Brosnan and Evans, unpublished) that the extent of calmodulin-stimulation depends on the amount of calmodulin-binding protein in the assay.

Using the assay methods described in this chapter basal ATPase activities between

46 and 58 nmol min^{-1} (mg protein)$^{-1}$ were established. The extent of calmodulin-stimulation also varied between preparations, from 3.8 nmoles min^{-1} (mg protein)$^{-1}$ to 8.1 nmoles min^{-1} (mg protein)$^{-1}$.

The results presented in Figure 3.1 indicated that calmodulin-stimulation of Pi release was retained over a four hour time period. This persistence of calmodulin-stimulation provided further indication that the assay was reliable, and permitted large numbers of assays to be carried out within one experiment if necessary. Basal activity declined over this time period, but this was likely to represent a decline in either non-calmodulin-stimulated ATPase activity or non-specific phosphatase activity.

ii). Nucleotide specificity of inorganic phosphate release from microsomes.

Data presented for nucleotide specificity of Pi release (Figure 3.2) indicated the highest basal ATPase activity was obtained with ATP (1 mM). Calmodulin stimulation was also maximal with ATP. There was relatively high basal Pi release in the presence of GTP, UTP and ITP but calmodulin stimulation was lower with these three nucleotides (ITP 34% , UTP 18% , GTP 6%). However, given the low total calmodulin-stimulated activity and the limits of accuracy of the assay system, it may well be that stimulation by nucleotides other than ATP was negligible. In particular, stimulation by ITP in the presence of up to 0.1 μM calmodulin was negligible, the 33% stimulation observed occuring between 0.1 and 0.3 μM. This differing pattern of stimulation from that in the presence of ATP suggested that the apparent stimulation in the presence of ITP was unlikely to be due to activity of the calmodulin-stimulated ATPase.

No evidence of nucleotide specificity was presented either by Dieter and Marmé (1981b) or by Robinson *et al.* (1988) for calmodulin-stimulation of ATPase activity. Low nucleotide specificity was demonstrated in studies of non-calmodulin-stimulated calcium transport (see section 3:1). Lack of specificity in basal Ca^{2+} transport in these preparations may be due to the presence of a calcium transporter or transporters showing different nucleotide specificities to the calmodulin-stimulated component in the membrane

preparations and may suggest the presence of more than one type of plasma membrane calcium transporter in plants (see Chapter 7). As the spectrophotometric assay used here measured Pi released from any source, not just nucleotide triphosphate hydrolysis, apparent lack of specificity is likely to be a result of the activity of other phosphate-releasing enzymes in the preparation, and therefore of little relevance to the CSCPA. One way in which improved data on nucleotide specificity of the calmodulin-stimulated component of ATPase activity could be obtained would be to use the radioisotope assay. This was not possible because labelled nucleotide triphosphates (other than ATP) of the required high specific activity were not available. For this reason it was also not possible to assess the nucleotide specificity of the purified CSCPA.

For comparison, the plant plasma membrane proton pump showed a high substrate specificity for ATP (Vara and Serrano, 1982; Clement *et al.* , 1986). Studies on the membrane-bound erythrocyte calmodulin-stimulated ATPase have also shown that its activity was dependent on ATP, while ITP, CTP, GTP or UTP did not function as substrates (Rega *et al.* , 1973).

iii). Effect of sodium orthovanadate on basal and calmodulin-stimulated inorganic phosphate release by microsomes.

From the results presented in Figure 3.3 it can be seen that orthovanadate inhibited basal and calmodulin-stimulated ATPase activity. The I_{50} for inhibition of basal activity was about 5 μM, maximal inhibition being about 16% of the activity in the absence of orthovanadate. This inhibition of basal activity may be largely due to inhibition of ATPases including the proton-pumping ATPase and basal activity of the CSCPA. The remaining 84% of activity which was not affected by orthovanadate was probably a result of phosphatase activity. Sodium orthovanadate inhibited calmodulin-stimulated Pi release with an I_{50} value of about 20 μM; complete inhibition of calmodulin-stimulated activity was obtained at 100 μM.

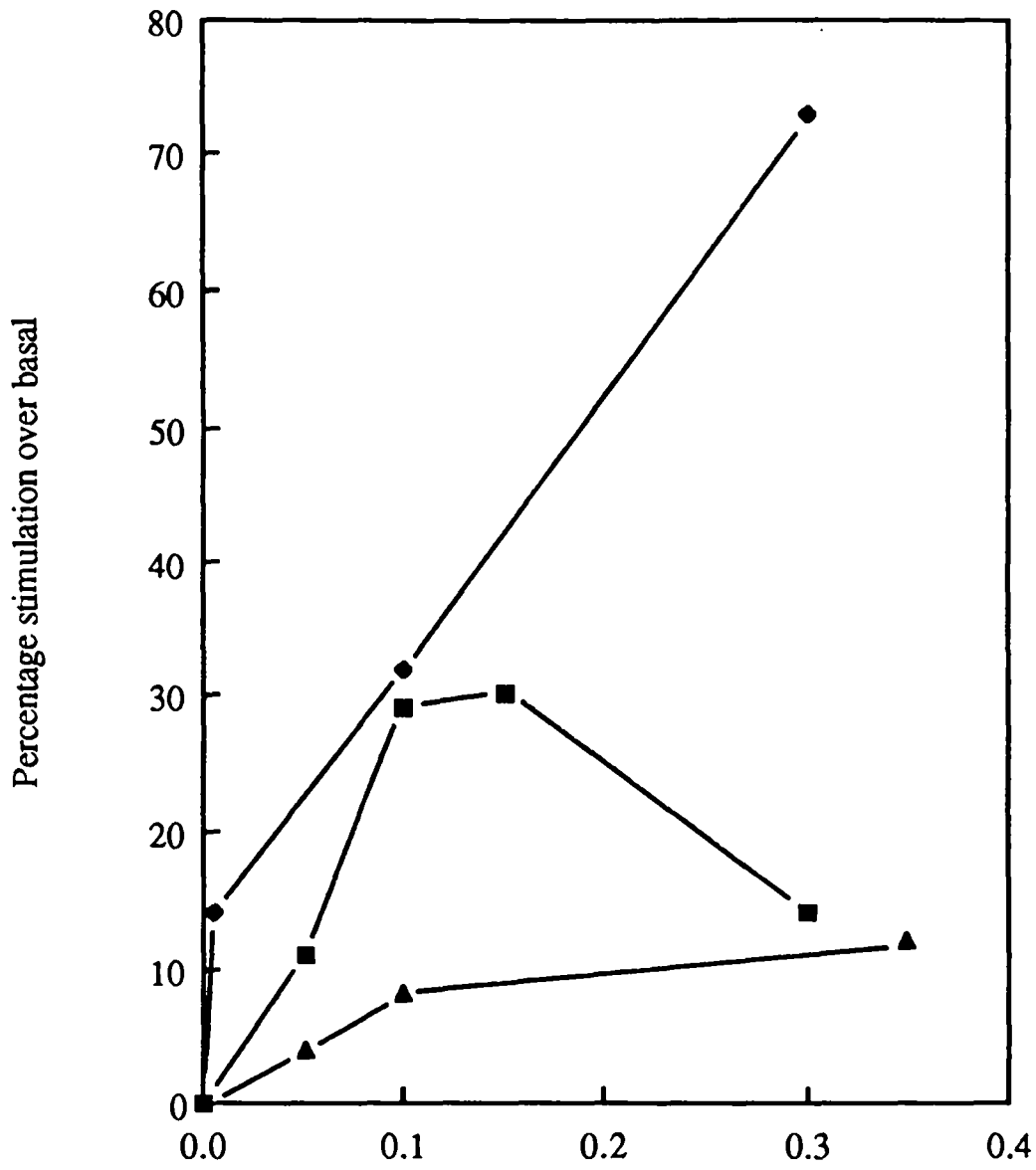
The effects shown here for the action of orthovanadate on basal ATPase activity

compare favourably with the report of Barrabin *et al.* (1980) who showed that in the presence of magnesium but not potassium (the conditions used for maize) the I_{50} value for orthovanadate inhibition of basal Ca^{2+}-ATPase activity in red blood cell membranes was about 25 μM or less. Neither of the two reports of calmodulin-stimulated ATPase activity in plant membrane fractions (Dieter and Marmé, 1981b; Robinson *et al.* , 1988) evaluated the effects of orthovanadate. Zocchi and Hanson (1983) showed that 100 μM orthovanadate inhibited 70% of bulk ATPase activity in partially purified plasma membranes, and Graf and Weiler (1989) reported bulk plasma membrane ATPase activity was inhibited by orthovanadate (I_{50}=10 μM). Previous reports on the effects of orthovanadate on microsome calcium transport have been conflicting; for example Dieter and Marmé (1980c) demonstrated that 20 μM orthovanadate had no effect while Butcher and Evans (1987b) showed 20 μM orthovanadate inhibited 70% of microsome calcium transport. The data presented in this chapter therefore suggest that Dieter and Marmé may have been wrong concluding that orthovanadate had no effect on calcium transport, a presumption which may have arisen from their failure to carry out a full dose/response experiment as was presented here and by Butcher and Evans (1987b).

3.3.2 Radioisotope assay for ATPase activity in microsomes

Use of the radioisotope assay for study of calmodulin-stimulated ATPase activity in maize microsomes yielded data that provided an interesting comparison with results from the spectrophotometric assay, as shown in Figure 3.4. Basal ATPase activity was lower in the radioisotope assay. This value was probably more representative of the ATPase activity of the microsome fraction, since the radioisotope assay measured only release of [γ^{32}P] from hydrolysis of [γ^{32}P]-ATP. Phosphate release measured in the spectrophotometric assay may originate from sources other than ATP hydrolysis. It is, however, difficult to explain why such large discrepancies in basal values occur between the two assays, given that the activity in the non-radioisotopic assay required

Comparison of calmodulin-stimulation of ATPase activity
(spectrophotometric assay ▲ , radioisotope assay ■)
and calmodulin-stimulation of calcium transport (◆)



the presence of ATP. It may be that it was due to phosphate release by non-specific phosphatases, the activity of which required ATP; or alternatively that it was due to contamination of the ATP or to release of the β-(and possibly α-) phosphate residues. Calmodulin-stimulation of [γ^{32}P] release was of the same order of magnitude as that of Pi release. It was found that the extent of calmodulin-stimulation in the radioisotope assay was more reproducible than in the spectrophotometric assay, being close to 8 nmoles min^{-1} (mg protein)$^{-1}$ in each experiment. As indicated above, there was a variation in maximal calmodulin-stimulated activity of between 4 and 8 nmoles min^{-1} (mg protein)$^{-1}$ using the spectrophotometric assay. Even with this extent of variation, the results from the two assays in terms of calmodulin-stimulation were sufficiently similar to give confidence that the effect of calmodulin in the spectrophotometric assay was due to stimulation of ATPase activity rather than stimulation of Pi release from other sources. It can be seen that the shape of the dose response curves from the two assays was not the same; in the spectrophotometric assay maximal stimulation was not achieved by 0.35 μM calmodulin, while the radioisotope assay showed that maximal stimulation occurred at about 0.1 μM. At concentrations above 0.1 μM stimulation by calmodulin declined in this assay. Reasons for this decline may lie in the nature of the radioisotope assay, possibly as a result of large amounts of Pi interfering with extraction efficiency.

The results in Appendix I confirm the work of Dieter and Marmé (1980d), showing that calmodulin-stimulated calcium uptake activity was also present in the microsome fraction of dark grown maize shoots. A calcium sensitive electrode was used to measure loss of free calcium from the incubation medium. The results obtained from the electrode traces showed that a linear rate of calcium uptake was obtained after the initial mixing period. It was found that the rates of calcium uptake varied between preparations: basal ATP dependent uptake ranged from 0.18-0.41 nmol min^{-1} (mg protein)$^{-1}$ (Table I.1), and a typical result for calmodulin-stimulated calcium uptake was 0.33 nmol

min^{-1} (mg protein)$^{-1}$, (1 μM calmodulin, Figure I.2). If percentage stimulation of calcium transport over basal was compared with the same value for both assays of ATP hydrolysis (see opposite) it may be seen that, up to 0.1 μM calmodulin, the radio-isotope ATPase assay closely resembled the calcium transport assay. Above this, the activity of the former declined for reasons discussed above. On the other hand, the phosphate release assay showed much lower percentage stimulation over basal throughout, due to the high basal values obtained. Another difference between the data obtained for ATPase activity and calcium transport was the approximately 25-fold discrepancy in activity. The range of values for calmodulin-stimulated Ca^{2+} uptake were 0.29 - 0.37 nmol min^{-1} (mg protein)$^{-1}$ as compared with a value for calmodulin-stimulated ATPase activity of about 8 nmol min^{-1} (mg protein)$^{-1}$ in the radioisotope assay . This difference may be explained in terms of the differences between the two assay systems. The radioisotope assay was carried out at 37°C and the calcium uptake assay at 25°C. In order to ensure that the vesicles in the microsome preparation were sealed for calcium transport, Triton X-100 was not included in the resuspension or incubation media. In the assay of calcium transport with the calcium electrode, only sealed vesicles orientated with the cytoplasmic face outwards would transport calcium; thus if a large proportion of microsome vesicles in this preparation were orientated with the cytoplasmic face inwards a lower rate of calcium transport would result. The requirement for potassium chloride in the incubation medium to maintain high ionic strength (and so permit a stable electrode reading) may have had the effect of bursting some of the vesicles, which would again lower the apparent rate of uptake. Comparison of basal calcium transport rates with those for ATP hydrolysis is less valid as the latter is likely to contain a number of enzyme activities not just those concerned with calcium transport (see above).

The dose response curve for the effect of varying amounts of calmodulin on calcium transport (Figure I.3) showed that maximal calmodulin-stimulated uptake occurred at 0.3 μM calmodulin. This profile was similar to that obtained for ATPase activity

measured by the spectrophotometric assay. It is interesting to note by comparison that the shape of the stimulation curve with added calmodulin for red blood cell membrane Ca^{2+}-ATPase activity with 17.4 μM free calcium (Foder and Scharff, 1981) was similar to that for the spectrophotometric ATPase assay presented in this chapter. It was also shown in Appendix I that addition of calcium ionophore A23187 to microsomes showing calmodulin-stimulated calcium uptake caused an immediate release of calcium into the medium. This confirmed that uptake (rather than binding) of calcium was occurring.

The studies presented in this chapter and in Appendix I have contributed to the characterisation of calmodulin-stimulated ATPase activity and calmodulin-stimulated calcium transport in microsomes from dark grown maize coleoptiles. A sensitive and reliable assay for release of ^{32}P from [$\gamma^{32}P$]-ATP was developed, which aided in the characterisation of calmodulin-stimulated ATPase activity and was suitable for use with purified CSCPA. The use of calcium sensitive electrode was a novel means of measuring calcium uptake by plant membrane vesicles; the results confirmed those of previous studies. As it was possible to measure calmodulin-stimulation of both ATPase activity and calcium transport, the foundation was laid for the detection and estimation of activity of the CSCPA during purification by calmodulin-affinity chromatography, which is described in the next chapter.

Chapter 4

PURIFICATION AND CHARACTERISATION OF THE PLASMA MEMBRANE CALMODULIN-STIMULATED, CALCIUM PUMPING ATPase OF MAIZE COLEOPTILES

4.1 INTRODUCTION

The results presented in the preceding chapter and Appendix I have confirmed and extended previous reports which demonstrated that microsome preparations from various plant tissues contained both calmodulin-stimulated ATP hydrolytic activity and

calmodulin-stimulated calcium uptake. Some properties of this activity were characterised and found to be consistent with the hypothesis that a component of microsomal calcium transport was brought about by the action of a calmodulin-stimulated, calcium pumping ATPase (CSCPA) probably located at the plasma membrane. Demonstration of calmodulin-stimulation of ATPase activity and calcium uptake in microsome fractions was an essential prerequisite for purification of the CSCPA by means of calmodulin affinity chromatography, the results of which are described in this chapter.

4.1.1 Principles of calmodulin-affinity chromatography

Affinity chromatography is a powerful technique for the purification of a variety of compounds. Its successful application lies in the identification of a specific ligand to the substance to be purified. The ligand is covalently attached to an inert chromatographic matrix and must retain its specific binding affinity for the substance of interest after it has been attached to the matrix. A mixture of components, including the substance to be purified, is applied to the affinity column and binding allowed to take place. In order to use the technique for membrane proteins they must first be solubilised from the phospholipid bilayer in a form which retains activity prior to application to the column. Next, unbound substances are removed by washing with a buffer that keeps the substances to be purified bound to the column but removes contaminants. Finally, the buffer is changed again to one which disrupts binding, preferably without inactivating or otherwise denaturing the compound of interest. Purity of the eluent will depend both on the number of compounds in the extract applied to the column which bind to the ligand and on the possibilities for specificity in elution conditions. Affinity chromatography has the advantage of concentrating as well as purifying the substance of interest. In addition, its use often permits the number of stages required to achieve purification to be reduced. This is of importance in the purification of integral membrane proteins, which are very labile when removed from their native phospholipid

environment. Calmodulin-affinity chromatography is a suitable method for purification of calmodulin-binding proteins because the binding of these proteins to calmodulin is usually calcium-dependent and reversible (Sharma *et al.* , 1983). In the presence of calcium, calmodulin-binding proteins bind to the calmodulin-affinity column, and are eluted when calcium is removed by washing the column with a buffer containing a calcium chelator such as EGTA or EDTA. This technique has been widely used for purification of the erythrocyte CSCPA (first reported by Niggli *et al.* , 1979b). Its use for purification of a plant CSCPA has been reported in one publication prior to the studies described in this thesis; Dieter and Marmé (1981b) solubilised microsomal membrane proteins with Triton X-100 and separated a calmodulin-binding fraction by affinity chromatography, which retained calmodulin-stimulated ATPase activity after elution and was suggested to represent the plant CSCPA. Use of calmodulin-affinity chromatography in the purification of the CSCPA from erythrocytes yields a pure product due to the high specificity and affinity of binding. It is possible that such a high degree of purity may not be achieved with plant membrane extracts due to the unknown affinity and specificity of binding of the plant CSCPA, and to the possible presence of other calmodulin-binding proteins in the extract. However, the results presented in this chapter demonstrate that the use of calmodulin-affinity chromatography is a suitable means of purifying the plant CSCPA.

4.1.2 Purification of the erythrocyte calmodulin-stimulated, calcium pumping ATPase

i). Purification of the erythrocyte CSCPA by means other than calmodulin-affinity chromatography; effects of phospholipids and detergents.

In the development of calmodulin-affinity chromatography as a suitable method for purification of the erthrocyte CSCPA, the establishment of four criteria were required, as below:

1. Identification of calmodulin as an activator of erythrocyte calcium transport and ATPase activity (Gopinath and Vincenzi, 1977; Jarrett and Penniston, 1977).

2. Development of suitable methods for solubilisation of the calcium transport and ATPase activity from the membranes in an active form (Wolf *et al.* 1977; Lynch and Cheung, 1979; Lichtner and Wolf, 1980a and b).

3. Development of suitable conditions for stabilising the solubilised CSCPA such that calcium transport and ATPase activities remained (Peterson *et al.* , 1978; Niggli *et al.* , 1979a; Gietzen *et al.* , 1980).

4. Demonstration that interaction of calmodulin with the calcium transporter in its solubilised form was direct and reversible (Niggli *et al.* , 1979a).

The criteria outlined above were established during early attempts to purify the erthrocyte CSCPA; in all of the reports cited above, purification was attempted by means other than calmodulin-affinity chromatography, and partial purification of the CSCPA was achieved. The main problems encountered in purification were the presence of band III protein (a contaminant in the preparation of Wolf *et al.* , 1976) and the very low abundance of the CSCPA in the membrane (estimated as less than 0.02% of total membrane protein, Knauf *et al.* , 1974).

ii). Purification of the erythrocyte CSCPA by calmodulin-affinity chromatography.

In the reports described, the four criteria required for the development of calmodulin-affinity chromatography as a means of purifying the erythrocyte CSCPA were shown to be met. The use of calmodulin-affinity chromatography to purify the erythrocyte CSCPA to near homogeneity was first reported by Niggli *et al.* (1979b). Triton X-100 (1 mg/mg protein) was used to solubilise membrane proteins, and after centrifugation to remove unsolubilised components, phosphatidylserine was added. The solubilised proteins were concentrated by ultrafiltration using a 100,000 M_r cut off filter before applying to the calmodulin-affinity column. Calmodulin was purified by standard techniques and coupled to cyanogen-bromide activated Sepharose 4B. The solubilised proteins were

applied to this column and unbound proteins removed by washing with a buffer containing calcium, phosphatidylserine and the protease inhibitor phenylmethysulphonyl fluoride (PMSF). Elution of the CSCPA was brought about by changing the column buffer to one containing EGTA, which caused elution of a small protein peak. This peak contained a high specific (Ca^{2+}+Mg^{2+})-ATPase activity, which was measured using a coupled enzyme assay. It was estimated that a minimum purification of 150-fold was achieved. It was noted, however, that added calmodulin did not stimulate the (Ca^{2+}+Mg^{2+})-ATPase activity, the reasons for which became clear in subsequent publications (see below). On SDS gel electrophoresis, three polypeptides were evident, a strongly staining polypeptide of approximate M_r 125,000 and two less strongly staining polypeptides of M_r 205,000 and 90,000. It was shown that the purified CSCPA formed phosphorylated intermediates in the presence of calcium at 205,000 and 125,000 M_r after incubation for short periods with [γ^{32}P]-ATP at 0°C followed by electrophoresis on pH 6.0 polyacrylamide gels. In the presence of EGTA, no phosphorylation was observed. From this work it was concluded that the intact CSCPA was of approximate M_r 125,000, and suggested that the polypeptide at 205,000 M_r was possibly a dimer, whilst that at 90,000 M_r was attributed to the presence of a small amount of band III.

iii). Reconstitution and characterisation of the erythrocyte CSCPA.

The work of Niggli *et al.* (1979b) referred to above (section ii) provided the basis upon which the erythrocyte CSCPA could be successfully purified and its properties characterised. In order to study it further it was necessary to develop methods by which the purified calcium transporter was incorporated into phospholipid vesicles of known composition. In the absence of phospholipids membrane transport proteins are very labile; it is possible to increase stability of these proteins by means of reconstitution. It was possible to investigate the properties and kinetics of the purified CSCPA after reconstitution. The aim of reconstitution is to provide an experimental system in which assays of the activity of purified membrane proteins can be made, and in which

studies of the reaction mechanism of the system can be carried out (Racker *et al.* , 1979). The principle of reconstitution is that vesicles of defined phospholipid content are produced by a variety of methods, and purified protein is incorporated into these vesicles. In the case of the erythrocyte CSCPA two methods have been described for achieving this purpose. Carafoli *et al.* (1980a) and Niggli *et al.* (1981a) incorporated the purified CSCPA into asolectin vesicles formed by sonication and passed the mixture through an abbsorbent column to remove the Triton X-100. Niggli *et al.* (1981a and 1982) also used a method modified from Kagawa and Racker (1971) in which asolectin, phosphatidycholine or phosphatidylserine vesicles were formed by sonication in the presence of cholate, and the purified ATPase was mixed with this, removal of the cholate and Triton X-100 present being brought about by an overnight dialysis step. In these reports it was maintained that Triton X-100 was removed by dialysis, although the low critical micelle concentration of Triton X-100 may prevent its complete removal. In order for successful reconstitution and measurement of ATPase activity to be carried out it is necessary that the CSCPA is inserted into liposomes with its cytoplasmic domain facing outwards and that the liposomes produced are sealed with respect to calcium and ATP. In general, procedures for reconstitution of membrane ion transporters remain empirical, it being necessary to determine the experimental conditions which permit demonstration of activity for each transporter.

A series of reports from the laboratories of Carafoli and Penniston have been published (for references, see below) which describe in detail the properties of the purified, reconstituted erythrocyte CSCPA. From these studies it is apparent that the erythrocyte CSCPA has certain characteristic properties. These may be summarised as below:

1. The erythrocyte CSCPA may be purified by calmodulin-affinity chromatography and in its intact form has an M_r of about 138,000 (this value being suggested to be more accurate by Graf *et al.* , 1982 than the original value of 125,000 M_r reported by Niggli *et al.* , 1979b).

2. The CSCPA may be reconstituted into liposomes of phosphatidlycholine and calmodulin-stimulated ATPase activity can be shown (Carafoli *et al.* , 1980b).

3. Reconstitution of the CSCPA into liposomes of acidic phospholipids (phosphatidylserine) caused stimulation of the ATPase activity; addition of calmodulin did not further enhance activity (Carafoli *et al.* , 1980a and b; Niggli *et al.* , 1981b).

4. After reconstitution of the CSCPA into asolectin vesicles (a lipid mixture containing phosphatidylcholine and other lipids) ATP-dependent calcium uptake could be demonstrated by several techniques (Carafoli *et al.* , 1980a; Niggli *et al.* , 1981b).

5. The purified CSCPA showed a high affinity for calcium (Carafoli *et al.* , 1980a and b; Niggli *et al.* , 1981a); the affinity of CSCPA for calcium was reduced by calmodulin or phosphatidylserine.

6. Purified CSCPA showed the properties expected of P-type ion translocating ATPases in that a phosphorylated intermediate was formed at 140,000 M_r by a calcium-dependent process (Niggli *et al.* , 1979b).

7. Vanadate inhibited the ATPase activity of the reconstituted CSCPA, half maximal inhibition occuring at 0.5 μM (Niggli *et al.* , 1981a).

8. After controlled digestion of the purified CSCPA with trypsin, a characteristic pattern of proteolysis products was formed (Graf *et al.* , 1982).

Subsequent to the studies cited above which established the fundamental properties of the CSCPA, much further work has been carried out, culminating in the determination of the amino acid sequence of parts of the CSCPA (for example, Filoteo *et al.* , 1987; James *et al.* , 1987, 1988) and determination of the cDNA sequence for the entire CSCPA (Verma *et al.* , 1988). Several reviews have been published on the properties of the erythrocyte CSCPA, for example, Carafoli and Zurini (1982), Carafoli *et al.* (1982), Schatzman (1982), Penniston (1983), Rega and Garrahan (1986), Niggli *et al.* (1987), Penniston *et al.* (1988). Further consideration of some of the properties of the erythrocyte CSCPA of relevance for comparative purposes to the results in this chapter

concerning the purification and properties of the maize CSCPA are presented in the discussion (section 4:3).

4.1.3 Purification of calmodulin-stimulated ATPase activity from maize coleoptiles by calmodulin-affinity chromatography

As indicated in Chapter 1, several studies (for references, see Chapter 1) have demonstrated the presence of calmodulin-stimulated ATPase activity and calcium uptake in microsome and plasma membrane fractions from various plant species (see also Chapter 2 and Appendix I). However, in only one study prior to the work described in this chapter (Dieter and Marmé, 1981b) was further purification of calmodulin-stimulated ATPase activity from plant cells carried out. It is suggested that the high affinity ($Ca^{2+}+Mg^{2+}$)-ATPase reported by Carter and Tipton (1987) may not be the same as the calmodulin-stimulated ATPase reported by Dieter and Marmé (1981b) and described in this chapter; further comment on this may be found in Chapter 1. The methods used by Dieter and Marmé (1981b) were similar to those described by Niggli *et al.* (1979b). The procedure used by Dieter and Marmé for extraction of microsomal membranes from etiolated *Zea mays* L. coleoptiles has been described (Chapter 3). Further purification was carried out by Dieter and Marmé (1981b) as follows. Membrane proteins from the microsomes were solubilised with 1 mg Triton X-100/mg protein. After centrifugation to remove unsolubilised proteins a small amount of calmodulin-stimulated ATPase activity could be detected in the supernatant. This was then applied (without concentration) to a calmodulin-sepharose affinity column. Unbound proteins (which contained a large amount of ATPase activity, but no calmodulin-stimulated activity) were washed from the column with a buffer containing 10% w/v) sucrose, 25 mM MOPS titrated to pH 7.5 with solid Tris, 0.5 M NaCl, 5 mM $CaCl_2$, and 0.05% w/v) Triton X-100. After washing the column with 2-3 bed volumes of this buffer, calmodulin-binding proteins were eluted with a buffer of the same composition as above, except that $CaCl_2$ was replaced

with 10 mM EGTA. The EGTA-eluted fraction was brought to 15 mM final calcium concentration and was shown to be stimulated two-fold by 1 μM added calmodulin. Calmodulin-stimulation in the partially purified extract was half maximal at about 0.2 μM, and maximal at 1 μM; addition of the calmodulin antagonist fluphenazine (to 50 μM final concentration) caused total inhibition of activity stimulated by 1 μM calmodulin. There was a relatively high level of calmodulin-independent ATPase activity in this fraction, which was suggested by the authors to represent either another ATPase which co-purified with the calmodulin-sensitive ATPase and was insensitive to calmodulin after affinity chromatography, or to arise because the calmodulin-sensitive ATPase had a basal calmodulin-independent activity. In the work of Dieter and Marmé (1981b), no protease inhibitors such as DDT or PMSF were included in the purification procedure, and the partially purified calmodulin-stimulated ATPase was assayed in the absence of phospholipids and without reconstitution. This procedure formed the basis for purification of the CSCPA from maize coleoptiles described in this chapter; as described in Chapter 3 some modifications to the method of Dieter and Marmé were required before an active microsomal preparation could be prepared.

The results presented in this chapter are the first to document purification of the intact CSCPA from maize coleoptiles and to demonstrate that the intact CSCPA was recognised by an affinity-purified polyclonal antibody raised against the erythrocyte CSCPA (see also Briars *et al.* , 1988). This indicates that there is homology between the two calcium transporters. In addition it was possible to establish that some of the properties of the maize CSCPA were similar to those of the erythrocyte CSCPA (as described above). In this study, calmodulin-stimulated ATPase activity in both non-reconstituted and reconstituted samples of the CSCPA was demonstrated using the radioisotope assay described in Chapter 2, section 2:2:4, the principle of which was described in Chapter 3. Information was also obtained concerning possible proteolysis of maize CSCPA and the subunits to which calmodulin bound (described in Chapter 2,

sections 2:3, 2:4:1; 2:2:8). Formation of phosphorylated intermediates by the purified CSCPA is described in Chapter 5. A collaboration was also initiated during the course of this study with Professor E. Carafoli, Eidgenössiche Technische Höchschule, Zürich, with the aim of determining the cDNA sequences of the maize CSCPA; preliminary data concerning amino acid sequences of short regions of the maize CSCPA as compared with those of the erythrocyte CSCPA are presented in Appendix II.

4.2 RESULTS

4.2.1 Purification and reconstitution of the CSCPA by calmodulin-affinity chromatography in the absence of PMSF: assay for calmodulin-stimulated ATPase activity and SDS-PAGE of fractions

i). Purification without phospholipids.

Following the sucessful demonstration of calmodulin stimulation of calcium transport and ATPase activity in microsomes described in the previous chapter and Appendix I, it was decided to attempt purification of the CSCPA by means of extracting microsomes by the method described (Chapter 2, section 2:2:2 and Chapter 3, section 3:2:1) followed by solubilisation and purification as described by Dieter and Marmé (1981b). Details of the methods used for purification have been described (Chapter 2, section 2:2:3, and section 4:1:3 of this chapter). The column buffers used did not contain asolectin, and PMSF was not present in the extraction and column buffers. Assay of ATPase activity was carried out in samples from the affinity column using the radioisotope ATPase assay described in Chapter 2, section 2:2:4 and Chapter 3.

a) Purification after a two-hour column wash.

In the report of Dieter and Marmé (1981b) it was stated that the affinity column was washed with 2-3 bed volumes of the buffer containing calcium after application of the solubilised microsome proteins. In the experiments described in this chapter, the calmodulin-affinity chromatography column was routinely washed with the calcium-containing buffer (Buffer C)for two hours, until the A_{280} reading of the eluent returned to the value of the wash buffer. This represented a minimum wash of 10 bed volumes. Figure 4.1 shows a representative column profile, as measured by absorbance at 280 nm. The relatively high absorbances at 280 nm of Buffer C (as shown by the absorbance value of 1.1 after removal of unbound proteins) and D (after elution of the peak) were caused by the presence of Triton X-100 in these buffers. Triton X-100 absorbs strongly at 280 nm because of the presence of a phenyl group. Shortly after the change to Buffer

D calmodulin-binding proteins were eluted in a small peak as measured by absorbance at 280 nm. The fractions with absorbance values greater than the buffers were pooled and are subsequently referred to as the 'peak' fraction. In this figure, the location of fractions immediatly before and after the peak is indicated. These are subsequently referred to as pre- and post peak fractions, and were used as controls in some of the experiments described below. The amount of protein (as measured by a modified Lowry protein assay, Bensadoun and Weinstein, 1976) in the fractions throughout a representative purification procedure is shown in Figure 4.2. The total amount of protein in the peak fraction was about 0.3 mg. From these values it can be seen that there was apparently a very small amount of protein in the pre-and post-peak fractions. Figure 4.3 shows the total ATPase activity of some of the column fractions, measured by the radioisotope ATPase assay. There was a large amount of ATPase activity in the unbound fractions (total activity in fractions 1-15 of 3.53 nmol min^{-1}). Most of the ATPase activity of the solubilised fraction applied to the affinity column was removed by the washing process. The total ATPase activity in the fractions eluted by EGTA was lower, being in the region of 0.45 nmol min^{-1} for the four fractions of the peak in this representative experiment. It proved very difficult to establish a true 'blank' level for 'ATPase activity' in the absence of CSCPA. In most experiments, Buffers C and D affected this value to different extents, and as the eluted fraction lay in the change over between the two buffers it was not possible to establish a blank value for each fraction. The data presented in Figure 4.3 is representative of one of the few experiments in which basal (i.e. not calmodulin-stimulated) ATPase activity was high enough for the varying buffer effects to be of less significance and a clear peak of activity to be observed. Activity in the pre- and post-peak fractions may therefore be real (i.e. due to small amounts of non-bound protein or CSCPA running immediately before and after the main peak) or artefactual (due to the buffer effects described above).

Figure 4.4a represents more frequently obtained data from an experiment where

Buffers C and D had a greater effect on apparent 'basal' activity. The low basal value for the eluted peak is presumably the effect of material (e.g. protein and Triton X-100) eluted from the column with the CSCPA on the assay. However, it was apparent that the eluted peak fraction contained CSCPA as it was clearly stimulated by calmodulin (Figure 4.4b) whereas adjacent fractions were not. In view of the effects of buffer on blank values, the basal ATP hydrolytic activity becomes largely meaningless. In consequence, subsequent data in this section is presented as 'calmodulin-stimulated' activity (i.e. taking the value in the absence of calmodulin as zero). This unsatisfactory situation was finally remedied when CSCPA activity was assayed after purification by an 18 hour column wash in the presence of asolectin (Figure 4.10 see below). It can be seen in Figure 4.4b that in the presence of increasing amounts of calmodulin, the ATPase activity of the peak fraction reached a maximal value at about 0.1 μM added calcium.

Figure 4.5 shows calmodulin-stimulated ATPase activity in the peak fraction (data being taken from a different experiment to that in Figure 4.4b), expressed in terms of specific activity. It can be seen that in the presence of increasing amounts of calmodulin the ATPase activity increased steadily. Half maximal stimulation by calmodulin occured at about 0.07 μM calmodulin, although over the range of calmodulin concentrations used in this representative experiment a plateau of stimulation was not clearly shown. The maximum specific activity of the calmodulin-stimulated component (at 0.3 μM added calmodulin) in the peak fraction was 340 nmoles min^{-1} (mg protein)$^{-1}$ in this experiment, and of similar magnitude in the replicate experiment.

Samples of the non-retained, pre-peak, peak and post-peak fractions from the affinity column after a two hour wash were prepared for SDS polyacrylamide gel electrophoresis. In order for any bands to be detected on the gel (data not shown) it was necessary to develop the silver stain (Morrissey, 1981) for a long period, during which time the background of the gel became dark. Even after such treatment, there was insufficient

contrast between the bands and the background, rendering the gel unsuitable for photography. It was evident that a means of concentrating the protein was required before determination of the M_r values of the proteins in the peak fractions could be made. One way in which this was attempted is shown in Figure 4.6, where a sample of the EGTA eluted peak was concentrated in a microconcentrator (Amicon centricon 10, 10,000 M_r cut-off). The time required to reduce the volume of the sample to a quarter that of the original volume was 60 minutes. After silver staining a strongly stained band at approximately 40,000 M_r was apparent. A further band was also visible at a slightly higher M_r value. Further attempts at SDS polyacrylamide gel electrophoresis of the purified fraction are described after improvements in techniques for purification and electrophoresis (section 4:2:2).

b) Purification after an 18 hour column wash.

The results presented in the section above for calmodulin stimulation of ATPase activity in the peak fraction compared favourably with those of Dieter and Marmé (1981b), as discussed in ~~Results~~ section 4:3:1. However, in the studies presented in this chapter attempts were made to include more controls in the assays for ATPase activity. From the data presented in the preceding section, it was apparent that difficulties were encountered in obtaining satisfactory values for the pre-peak, post-peak and buffer only controls. A series of modifications to the purification procedure described above were made after reference to the literature concerning purification of the erythrocyte CSCPA in order to investigate whether it was possible to improve on these results.

One such modification was to wash the calmodulin-affinity column for 18 hours with the calcium containing buffer (Buffer C); this was reported by Graf *et al.* (1982) to be a means of removing contaminants from the CSCPA of erythrocytes purified by calmodulin-affinity chromatography. This was attempted in the purification of the maize CSCPA using the same buffers as in the preceding section (4:2:1ia). As shown in Figure 4.7 the amount of protein eluted by Buffer D after the overnight wash was very

low on the basis of absorbance at 280 nm. The amount of protein in this peak fraction was measured by the protein assay of Bensadoun and Weinstein (1976); a value of 0.09 mg total protein was found. The peak fraction was assayed for calmodulin-stimulated ATPase activity, but no evidence was found for calmodulin stimulation over a range of calmodulin concentrations from 0-0.3 μM (ATPase activity without calmodulin, 260 ($\pm$18) nmoles min^{-1} (mg protein)$^{-1}$; ATPase activity with 0.3 μM calmodulin, 260 ($\pm$23) nmoles min^{-1} (mg protein)$^{-1}$).

ii). Reconstitution of purified CSCPA.

Reconstitution of the purified erythrocyte CSCPA was carried out soon after it had been purified, and was first reported by Carafoli *et al.* , 1980a and b. The aim of reconstitution in the study of membrane ion transport is to insert purified transporter into phospholipid liposomes such that activity can be measured. Reconstitution of the maize CSCPA had not been attempted prior to the work described in this chapter, and so the methods used were derived from the literature concerning reconstitution of erythrocyte CSCPA. This has been reconstituted by two main methods; cholate-dialysis and by removal of Triton X-100 with a Bio-Beads SM2 column. It was suggested by Professor E. Carafoli (pers. comm.) that use of cholate dialysis into asolectin vesicles was the most appropriate technique for demonstration of the maize CSCPA. A series of reconstitution experiments were carried out with the maize CSCPA purified by means of the two hour column wash as described above, with the aim of demonstrating ATPase activity, and investigating the effects of calmodulin.

The purified CSCPA was reconstituted into asolectin vesicles by the cholate dialysis technique as described in Methods 2:2:7, which was a method taken from Niggli *et al.* (1987). The asolectin preparation used in these experiments was from Sigma, being described as L-α-phosphatidycholine, type II-S from soybean, with a phosphatidylcholine content of 10-20% . After overnight dialysis, the total amount of protein remaining in the peak fraction, as measured by the protein assay of Bensadoun and Weinstein (1976),

was 0.1 mg, as compared with 0.3 mg before dialysis. The pre-peak, peak and post-peak fractions were assayed for calmodulin-stimulated ATPase activity, and it was found that, as previously, the pre- and post-peak and buffer controls were greater than the peak sample. In these control samples there was no evidence for calmodulin stimulation of apparent ATPase activity. The data presented in Figure 4.8 shows calmodulin stimulation of ATPase activity in the peak fraction reconstituted into asolectin, expressed as calmodulin stimulation over the apparent activity in the absence of calmodulin. It appears that maximal calmodulin stimulation of ATPase activity occured at about 0.025 μM calmodulin, remaining maximal between 0.025 μM and 0.2 μM. At 0.3 μM calmodulin, the extent of stimulation had apparently declined. The maximal value for calmodulin stimulation was about 500 nmoles min^{-1} (mg protein)$^{-1}$.

iii). Purification with asolectin in the column buffers.

As described above in sections i. and ii. difficulties were encountered with assays of ATPase activity in the purified CSCPA and the reconstituted CSCPA in that values for the buffer blanks were higher than those of the peak fraction. Further modifications of the purification procedure were carried out in order to improve on these results and so obtain more reliable estimates of ATPase activity of the purified CSCPA. Purification of the erythrocyte CSCPA as described by Niggli *et al.* (1979b) was carried out in the presence of phospholipids in the column buffers. The procedure described in section 4:2:1 above to purify the maize CSCPA was modified as follows. A suspension of asolectin (to final concentration of 20 mg/ml) was added to the Triton X-100 solubilised supernatant from the 100,000xg centrifugation. Asolectin (0.1%)was sonicated into the column buffers and the column was washed for 18 hours with Buffer C containing asolectin prior to elution of calmodulin-binding proteins with Buffer D. The protein profile of this purification procedure as measured by absorption at 280 nm is shown in Figure 4.9. In this column profile, the inclusion of asolectin in the column buffers caused the absorbance of these buffers to be increased to about 2.2 A_{280} units,

as compared with about 1.1-1.3 units for buffers without asolectin. It was also found that the absorbance values of Buffers C and D were not always the same when asolectin was added; this probably occured as a result of different amounts of asolectin having been sonicated into the two buffers, although the same concentration of asolectin was added to each buffer initially, and these were sonicated and filtered simultaneously in each experiment. However, an EGTA eluted peak was always detected. The amount of protein in the peak fraction purified by this method was estimated as 0.09 mg by the assay of Bensadoun and Weinstein (1976). Pre-peak, peak, post peak and Buffers C and D were assayed for ATPase activity, and it was found that these had lower values than peak fractions. It was therefore possible to make a correction for the apparent 'ATPase activity' in the buffer samples in the calculation of specific activity for the peak fraction. The result obtained is presented in Figure 4.10, in which the values have been corrected by subtraction of c.p.m. in the pre-peak fraction before conversion to specific activity. This method of calculation indicated that there was still some ATPase activity (about 80 nmoles min^{-1} (mg protein)$^{-1}$) in the peak fraction that was calmodulin-independent. In these experiments the range of calmodulin concentrations over which ATPase activity was measured was reduced to 0-0.05 μM, since in the experiment in which the CSCPA was reconstituted into asolectin, maximal calmodulin stimulation apparently occured at 0.025 μM. The maximal stimulation of ATPase activity by calmodulin was about 100 nmoles min^{-1} (mg protein)$^{-1}$ which occurred at about 10 nM calmodulin.

Samples of the pre-peak, peak and post-peak fractions from the column run depicted in Figure 4.9 were concentrated by precipitation with TCA prior to electrophoresis on neutral SDS polyacrylamide gels. The gel from this experiment was divided into two, one half being stained with Coomassie Blue and the other with silver stain (Morrissey, 1981). Figure 4.11a (lane c) shows that, in the gel stained with Coomassie Blue, three polypeptides of M_r approximately 62,000, 66,000 and 72,000 were apparent in the peak sample concentrated with TCA. These were not visible in the peak sample which was

applied to the gel without concentration as shown in Figure 4.11a, lane b. In the pre- and post-peak samples (Figure 4.11a, lanes a and d) concentrated with TCA only very faint staining was apparent. When silver staining was used to detect the polypeptides a slightly different pattern of staining was apparent. In the peak sample concentrated with TCA (Figure 4.11b, lane c) only one polypeptide was clearly visible at M_r 72,000. No polypeptide were visible in the peak sample which had not been concentrated with TCA (Figure 4.11b, lane b). A sample of the pre-peak sample concentrated with TCA (Figure 4.11b, lane a) did not show any staining with silver stain. However, a polypeptide of about 72,000 M_r was visible in the post-peak fraction concentrated with TCA (Figure 4.11b, lane d).

Table 4.1 summarises the data obtained for ATPase activity after purification of the CSCPA by the methods described above. If comparison is made on the amount of protein in the initial homogenate as compared with the peak fractions, it can be calculated that the maximum purification factor (based on protein) was 10,700-fold for purification after an 18 hour column wash in the presence of asolectin. In the microsome preparation there was high total activity (both calmodulin-stimulated and basal) but low specific calmodulin-stimulated activity (8 nmol min^{-1} (mg protein)$^{-1}$). For reasons indicated above, it is only possible to compare calmodulin-stimulated ATPase activity in the different purification conditions. If the calmodulin-stimulated specific ATPase activity in microsomes is taken as a reference point, then the highest level of purification achieved was in the peak fraction eluted after two hour wash and reconstituted into asolectin vesicles. In this fraction the degree of purification achieved was about 70-fold. In the other fractions (peak eluted after a two hour wash and peak eluted after an 18 hour wash with asolectin) the apparent purification achieved was lower.

4.2.2 Purification of the maize CSCPA in the presence of PMSF: SDS-PAGE, immunoblotting and detection of calmodulin binding proteins

i). SDS-PAGE.

In the report of Gallagher and Leonard (1987) it was demonstrated that certain precautions were required in the preparation of samples of plasma membrane proteins for electrophoresis to avoid proteolysis and aggregation of these proteins. This was shown with reference to the proton pumping ATPase. In the report, plasma membrane fractions were prepared by discontinuous sucrose density gradient centrifugation and proteins were extracted by treatment with Triton X-100 or Triton X-114. This procedure was carried out with or without the protease inhibitors chymostatin (25 μg/ml) and PMSF (1 mM) and it was shown that resolution of the polypeptides on SDS-PAGE was improved when these protease inhibitors were included in the extraction. It was also shown that further improvement could be obtained if the solubilised membrane proteins were prepared for SDS-PAGE by being mixed in equal volumes with sample buffer (0.125 mM Tris-HCl, pH 6.8, 10% SDS, 0.2 mM DTT, 10% glycerol and 0.002% bromophenol blue) containing the protease inhibitors chymostatin (500 μ/ml) and PMSF (5 mM). It is usual during preparation of samples for SDS-PAGE for the samples to be incubated at 100°C after addition of the sample buffer in order to ensure reduction. In the report of Gallagher and Leonard (1987) however, it was shown that the best resolution of the proton-pumping ATPase (at 100,000 M_r) on SDS-PAGE was obtained with samples that had been extracted in the presence of protease inhibitors, taken up in sample buffer with protease inhibitors and which had not been boiled prior to electrophoresis. The calmodulin-affinity chromatography column buffers used by Niggli *et al.* (1979b) also contained PMSF. It was decided to modify the composition of the buffers used in the purification of the maize CSCPA by inclusion of 1 mM PMSF in all buffers throughout extraction and purification. The EGTA-eluted peak was precipitated with TCA in

'Eppendorf' tubes which had been treated with 'Repel-Silane' (Pharmacia-LKB) which prevented the protein from adhering to the plastic walls of the tubes. The precipitated proteins were taken up into sample buffer containing 500 μg/ml chymostatin and 5 mM PMSF and were incubated on ice for a minimum of 30 minutes prior to separation on SDS-PAGE. The results obtained have been presented by Briars *et al.* (1988) and are depicted in Figure 4.12. Lane a shows relative molecular mass markers (which were prepared by being taken up in SDS sample buffer without protease inhibitors, and were incubated for 3 minutes at 100°C). A sample of microsome protein was also electrophoresed after preparation as for the purified sample (lane c). Lane b shows the separation of proteins eluted in the peak fraction from the calmodulin-affinity chromatography column. The gel was 7.5% polyacrylamide, 0.2% bis-acrylamide and was stained with Coomassie Blue (Chapter 2, section 2:3:3). A polypeptide of M_r 140,000 ($\pm$10,000 M_r) and other polypeptides at 91,000, 77-69,000, 51,000 and 40,000 M_r were evident. The relative molecular mass of these polypeptides was verified by carrying out electrophoresis several times on 7.5% and 5% SDS polyacrylamide gels. The M_r values were also verified by electrophoresis on pH 6.0 SDS polyacrylamide gels (see Chapter 5).

ii). Immunoblotting.

In order to investigate possible similarity between the CSCPA purified from maize and that from erythrocytes, a sample of the maize CSCPA was prepared as described above (section i). The entire peak fraction was concentrated by TCA precipitation and then divided into three before electrophoresis on a 7.5% SDS polyacrylamide gel. Each lane with purified CSCPA contained a maximum of 100 μg of protein, as measured by the assay of Bensadoun and Weinstein (1976). The gel was divided such that lanes containing M_r markers, microsome protein and purified CSCPA were stained with Coomassie Blue, as shown in lanes a, b and e of Figure 4.13a. The proteins in the other part of the gel (two lanes each of purified CSCPA and microsome proteins) were electrophoretically transferred to nitrocellulose membranes as described in Chapter 2,

section 2:4:1. Immunoblotting was carried out as described (Chapter 2, section 2:4:1) with the monospecific polyclonal antibody 97.1, raised by F. Kessler (Eidgenössiche Technische Höchschule, Zürich). This antibody was raised in rabbits against the erythrocyte CSCPA (Verma *et al.* , 1982) and affinity purified as described by Verma *et al.* (1984). A lyophilised sample of antibody 97.1 was taken up in 0.5% (w/v) milk powder in PBSA. After incubation with 97.1 followed by visualisation with gold-conjugated secondary antibody and silver enhancement, it can be seen that the anti-erythrocyte CSCPA antibody recognised the 140,000 M_r polypeptide in the purified maize CSCPA, as shown in Figure 4.13a, lane c. This did not arise as a result of non-specific staining by the secondary antibody as no staining was apparent in a sample of purified CSCPA incubated without primary antibody 97.1 but otherwise processed in the same way (Figure 4.13a, lane d). Antibody 97.1 did not recognise any polypeptide in the microsome fraction, as shown in Figure 4.13a, lane f (with primary antibody) and lane g (without primary antibody). These results have been presented by Briars *et al.* (1988).

To show that antibody 97.1 was recognising the erythrocyte CSCPA, a sample of lyophilised erythrocyte ghost protein (supplied from Zürich) was taken up in SDS sample buffer (with protease inhibitors) and electrophoresed on a 7.5% SDS polyacrylamide minigel. The separated proteins were electrophoretically transferred to nitrocellulose membranes; one lane was stained for protein content using 'Aurodye forte' and the other was stained with antibody 97.1 as described above. Figure 4.13b shows the result obtained. In the erythrocyte ghosts, a range of polypeptides were visible (lane a); antibody 97.1 stained a band at 140,000 M_r (lane b) which was only very faintly visible in the erythrocyte ghost preparation.

iii). Localisation of calmodulin-binding proteins in purified CSCPA.

The purification procedure described in ~~Results~~ section 4:2:2i was used to purify the intact CSCPA from maize. The entire peak fraction was concentrated by TCA precipitation and applied to one lane of a 7.5% SDS polyacrylamide gel. The proteins

were electrophoretically transferred to nitrocellulose membranes, and stained with gold-conjugated calmodulin. The results obtained are depicted in Figure 4.14; the M_r values of the bands labelled with gold-calmodulin were 140,000, 91,000, 77-69,000, 51,000 and 40,000.

Figure 4.1: Calmodulin-affinity chromatography of Triton X-100 solubilised microsomes.

The sample (100KS) was applied to the column as indicated and the column washed with calcium-containing buffer (Buffer C, without PMSF or asolectin). The protein content of fractions (1.5 ml) collected from the column was measured by absorbance at 280 nm. The column was washed until the absorbance reading was that of Buffer C alone (1.1 A_{280} units). Calmodulin-binding proteins were eluted with EGTA-containing buffer. The fractions designated pre-peak, peak and post-peak in the text are indicated. Representative of seven experiments.

This method was used, with the modifications indicated in the individual figure legends, in Figures 4.7 and 4.9.

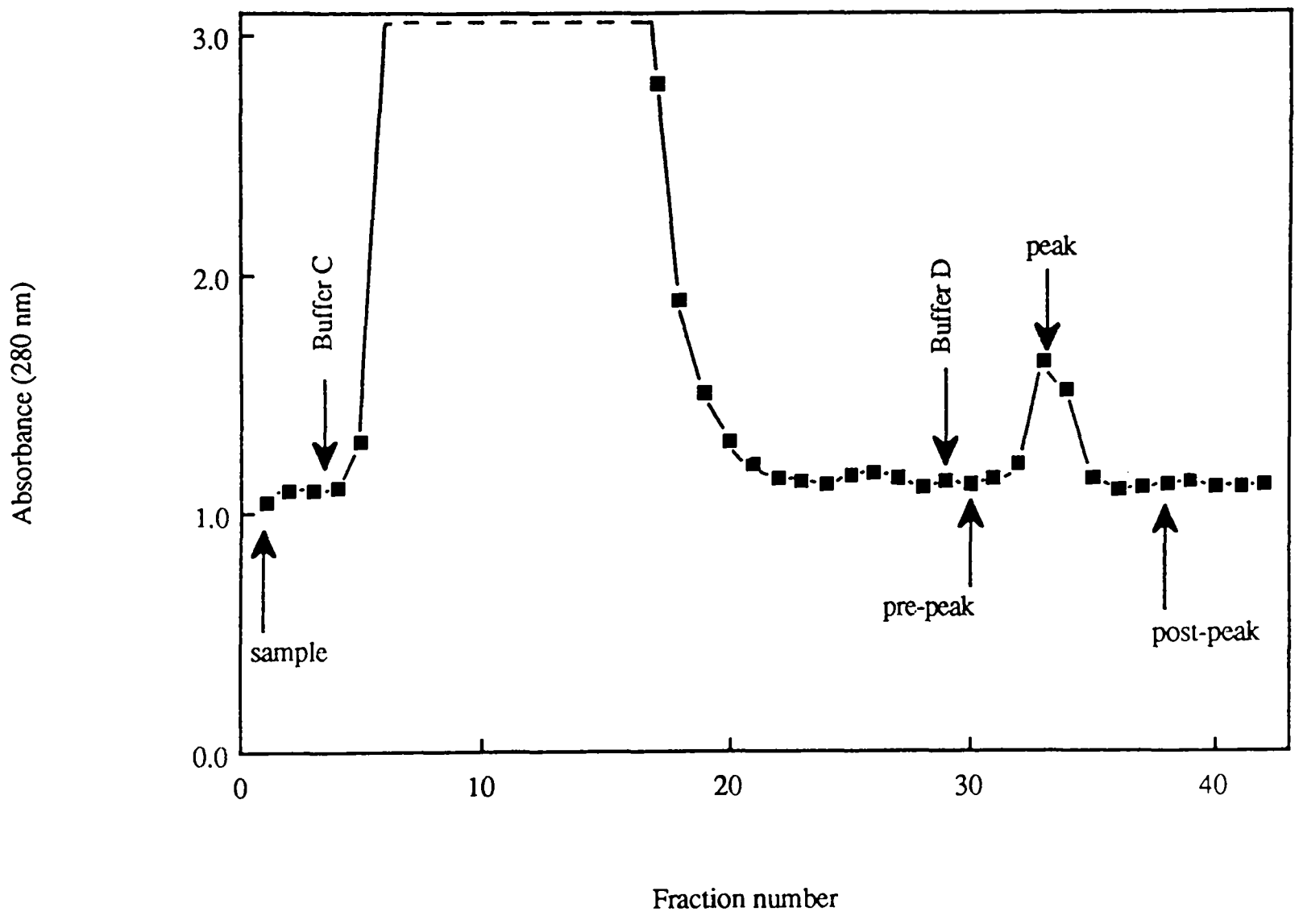

Absorbance (280 nm)
3.0
2.0
1.0
0.0
0
10
20
30
40
Buffer C
Buffer D
peak
sample
pre-peak
post-peak
Fraction number

Figure 4.2: Protein balance sheet for purification of maize calmodulin-stimulated, calcium-pumping ATPase by calmodulin-affinity chromatography.

Column buffers without phospholipids or PMSF, 2 hour column wash.

Representative of seven experiments; values are total amount protein (mg) in fraction except where otherwise indicated

PROTEIN BALANCE SHEET

Fresh weight of coleoptiles = 85g

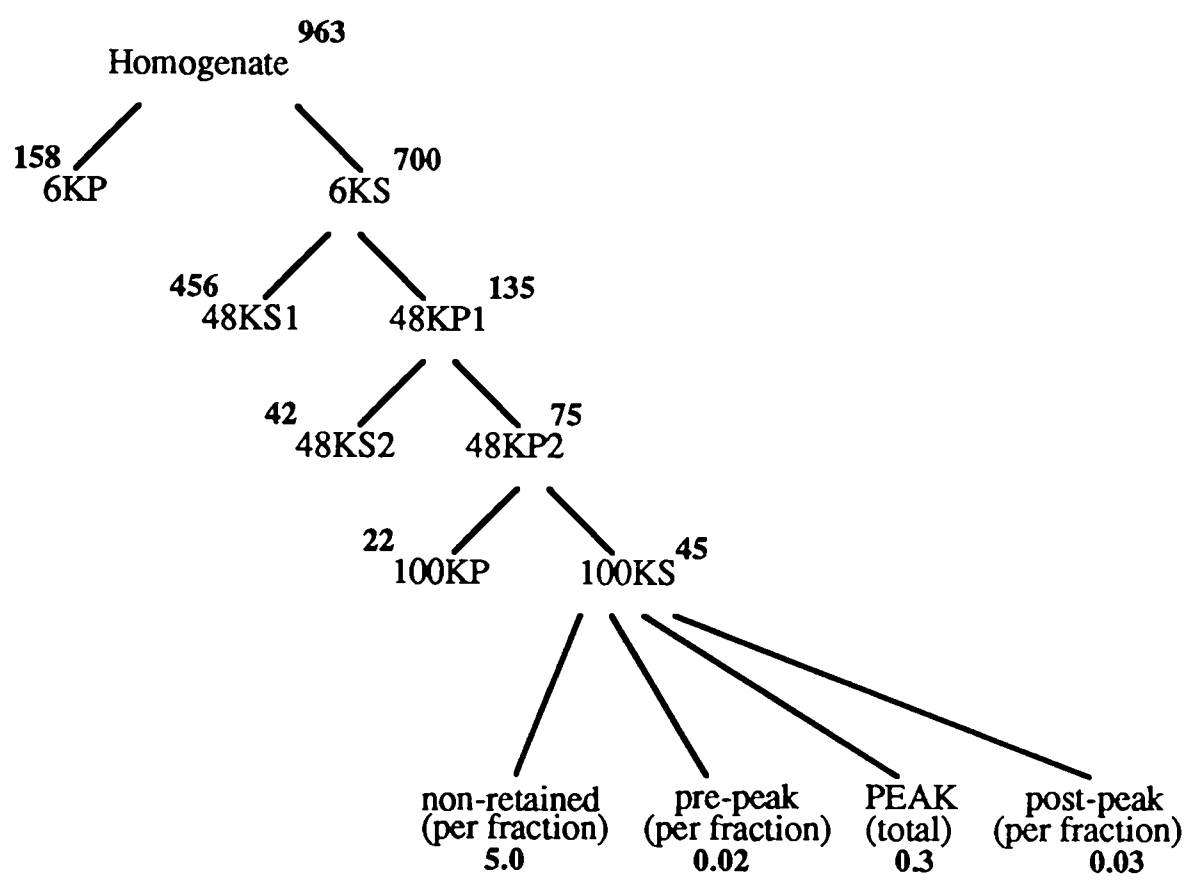


Figure 4.3: ATPase activity of fractions from calmodulin affinity column.

Fraction numbers correspond with those in Figure 4.1.

Radioisotope ATPase assay; values are means of triplicate determinations.

Data from one experiment; see text for details.

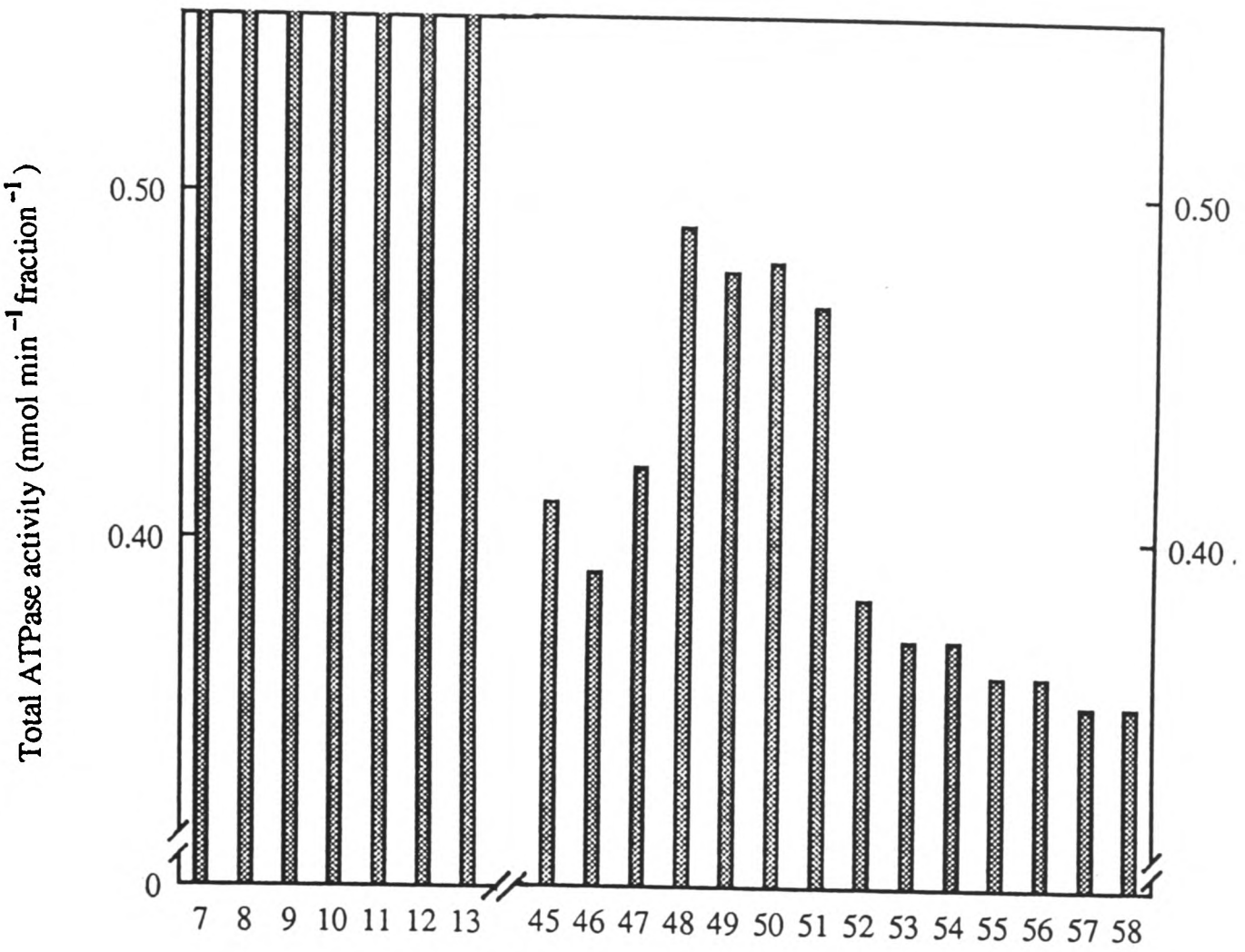

Total ATPase activity (nmol min^{-1} fraction^{-1})
0.50
0.40
0
0.50
0.40
7 8 9 10 11 12 13 45 46 47 48 49 50 51 52 53 54 55 56 57 58
Fraction

Figure 4.4

4.4a: Total ATPase activity (radioisotope assay) in fractions from calmodulin affinity column and Buffer D.

4.4b: Calmodulin-stimulated ATPase activity.

Activity in absence of calmodulin taken as zero.

■ peak

◆ pre-peak

▲ post-peak.

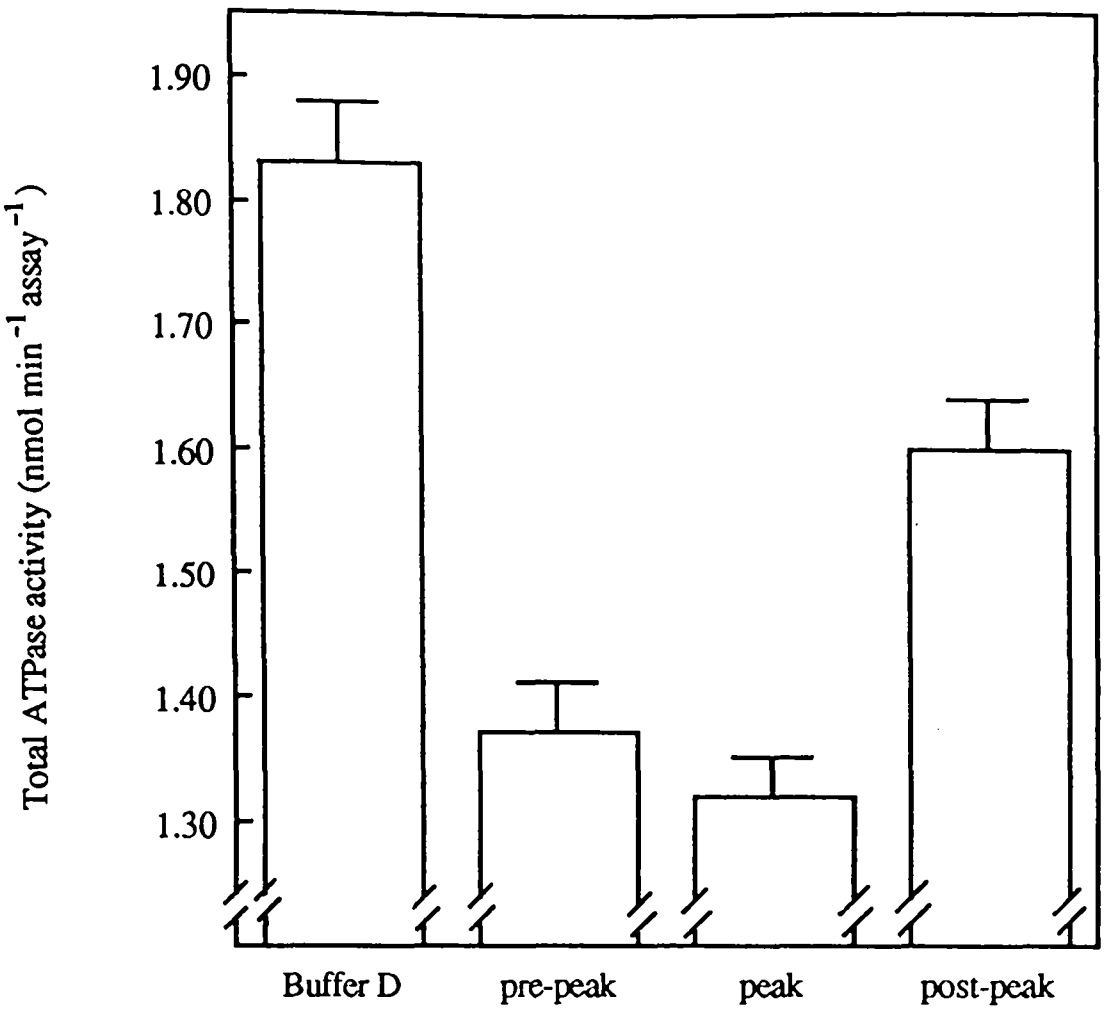
Values are means of triplicate determinations.

Representative of three experiments.

Protein content per assay: pre-peak 0.2 ng: peak 7 ng: post-peak 3 ng.

Calmodulin-affinity chromatography was carried out as described in Figure 4.1

4.4a


Total ATPase activity (nmol min^{-1} assay^{-1})
1.90
1.80
1.70
1.60
1.50
1.40
1.30
Buffer D
pre-peak
peak
post-peak

4.4b

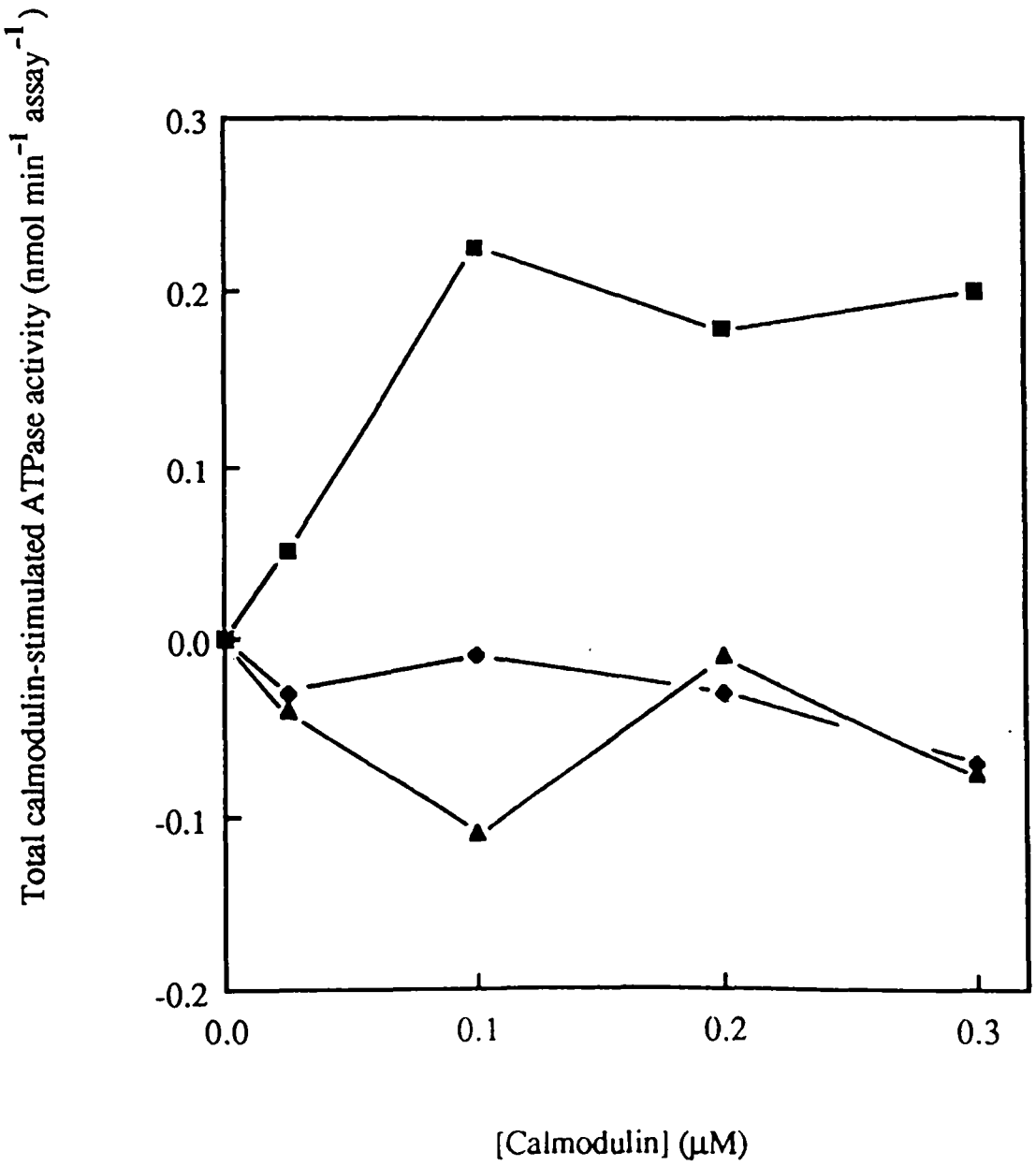

Total calmodulin-stimulated ATPase activity (nmol min^{-1} assay^{-1})
0.3
0.2
0.1
0.0
-0.1
-0.2
0.0
0.1
0.2
0.3
[Calmodulin] (μM)

Figure 4.5: Calmodulin-stimulation of ATPase activity in peak fraction eluted with EGTA following calmodulin-affinity chromatography (buffers without PMSF or asolectin, 2 hour column wash).

Radioisotope ATPase assay.

Representative of two experiments: values are means plus or minus one standard deviation (n = 3).

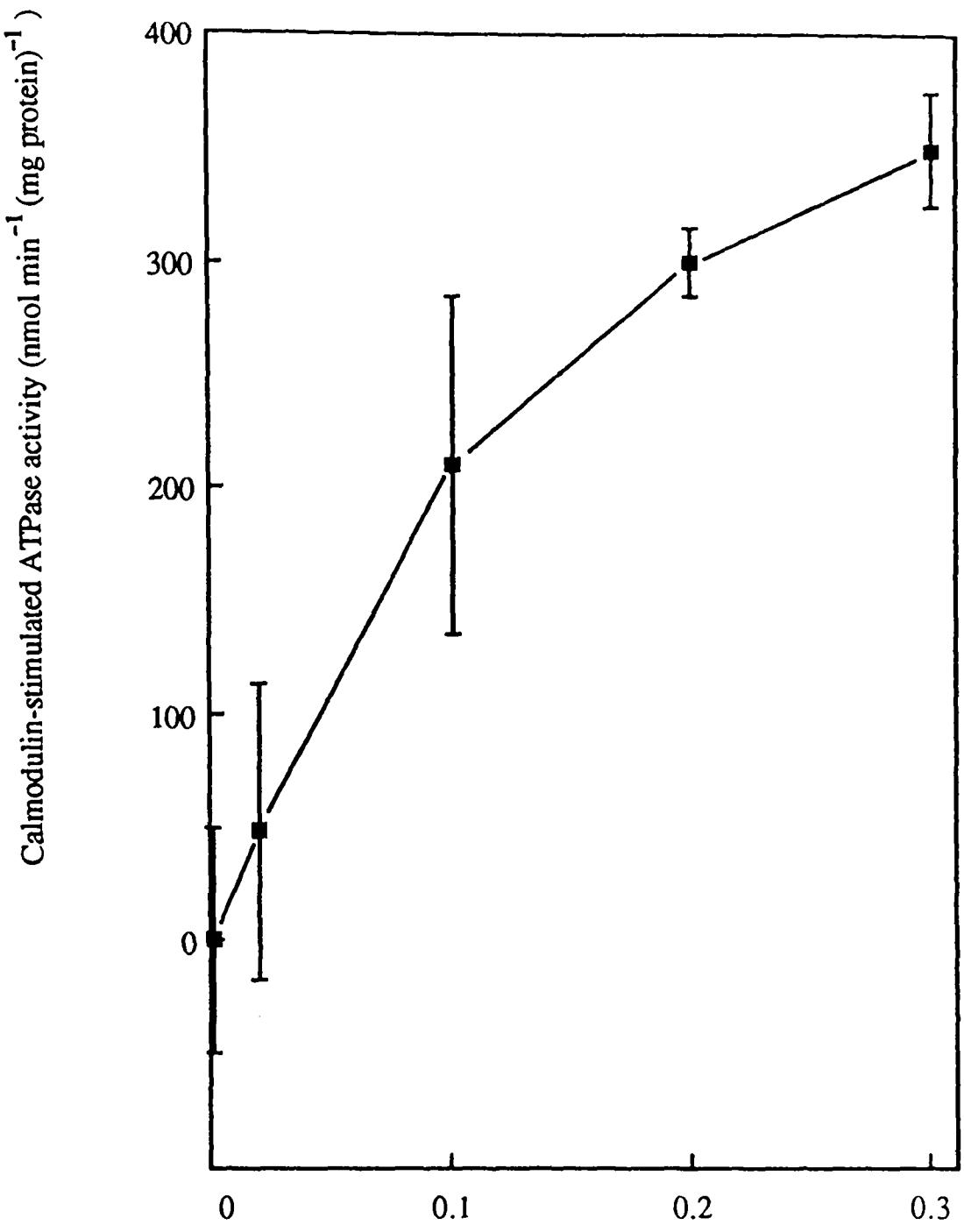

Calmodulin-stimulated ATPase activity (nmol min^{-1} (mg protein)$^{-1}$)
400
300
200
100
0
0
0.1
0.2
0.3
[Calmodulin] (μM)

Figure 4.6: SDS-polyacrylamide gel electrophoresis of EGTA-eluted peak fraction from calmodulin-affinity chromatography after microconcentration.

7.5% gel; purification in the absence of PMSF or asolectin, two hour column wash.

Calmodulin-affinity chromatography was carried out as described in Figure 4.1.

The peak fraction from the calmodulin affinity column was concentrated by centrifugation (5,000xg, 30 minutes) in 10,000 M_r cut off microconcentrators; the volume was reduced to about a quarter that of the original. The concentrated peak fraction was taken up in SDS sample buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.001% bromophenol blue, 10 mM DTT) and incubated at 100°C for 3 minutes. Discontinuous SDS-polyacrylamide gels were prepared (7.5% acrylamide, 0.2% bisacrylamide, ammonium persulphate as the polymerisation catalyst). Samples were applied to the wells of the gels and electrophoresis carried out at 200 V for 3-4 hours. Relative molecular mass markers were also separated on each gel.

The gel was stained with a sensitive silver stain (Morrissey, 1981). Gels were fixed in methanol and glutaraldehyde, washed thoroughly, and incubated in DTT. After incubation with silver nitrate, developer (0.05% of 37% formaldehyde and 0.3% sodium carbonate) was added. When sufficient staining had taken place, the reaction was stopped with citric acid and the gel photographed.

This method of electrophoresis was carried out (with the modifications noted in subsequent legends) in Figures 4.11, 4.12,4.13, 4.14, 6.1, 6.2, 6.3, and 6.4.

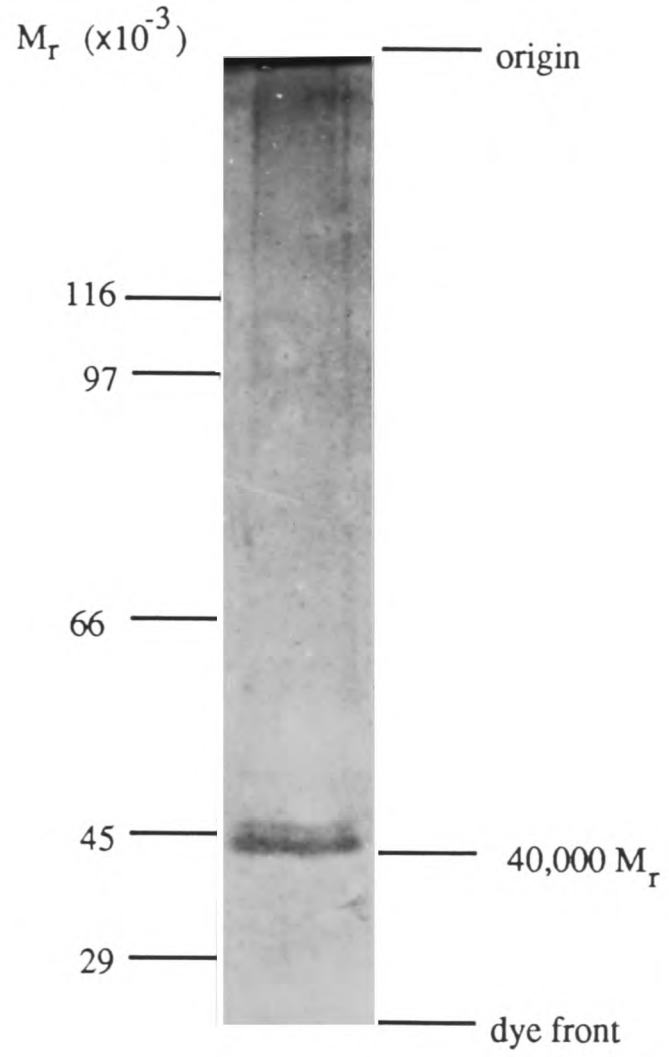

M_r ($x10^{-3}$)
origin
116
97
66
45
40,000 M_r
29
dye front

Figure 4.7: Calmodulin-affinity chromatography of Triton X-100 solubilised microsomes; 18 hour column wash without asolectin or PMSF in column buffers.

Protein content determined by absorbance at 280 nm.

Representative of two experiments.

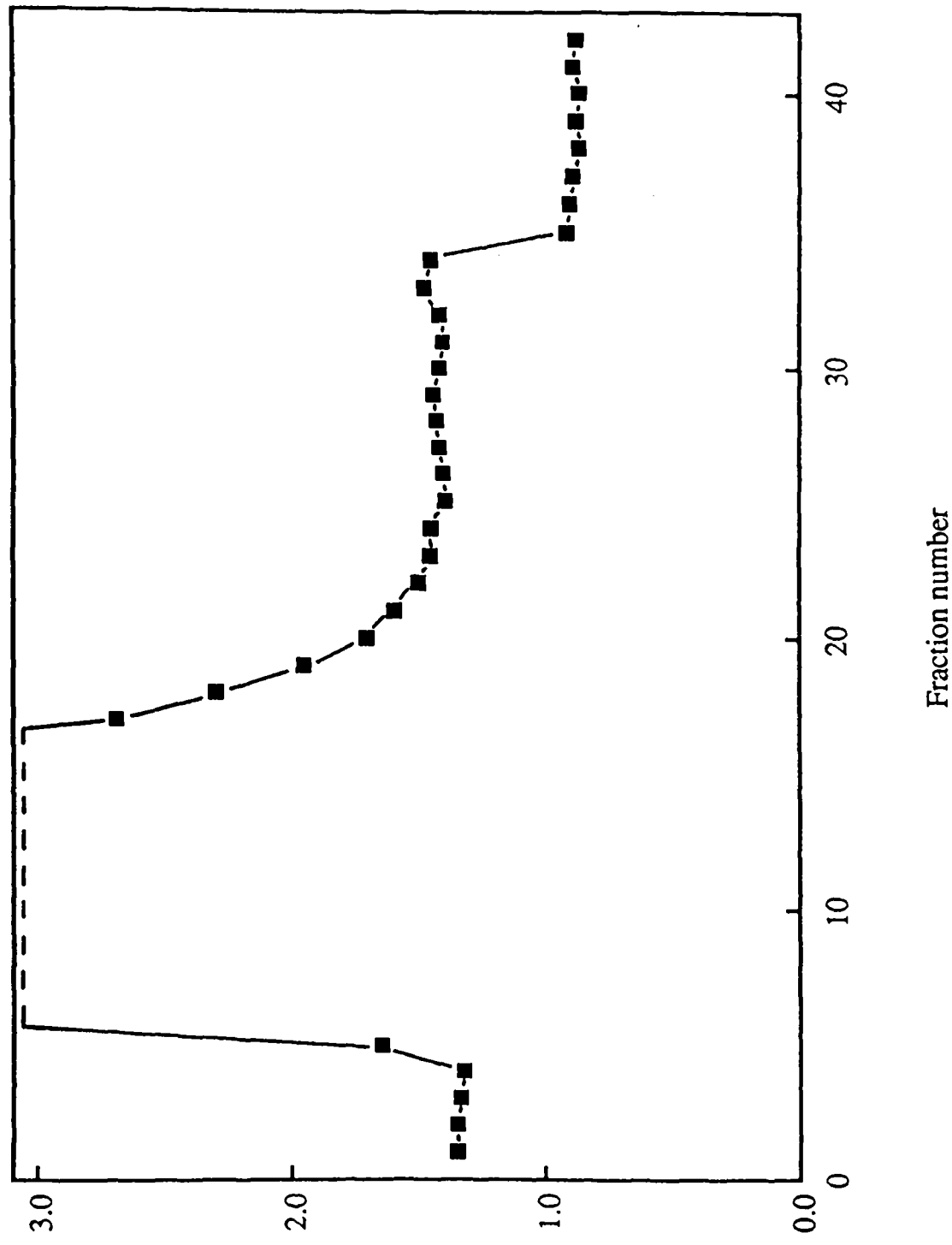

Absorbance (280 nm)
0.0
1.0
2.0
3.0
0
10
20
30
40
Fraction number

Figure 4.8: Calmodulin-stimulation of ATPase activity in EGTA-eluted peak from calmodulin-affinity chromatography after reconstitution into asolectin vesicles by cholate dialysis.

2 hour column wash without PMSF or phospholipids.

Representative of two experiments; values are means plus or minus one standard deviation (n = 3).

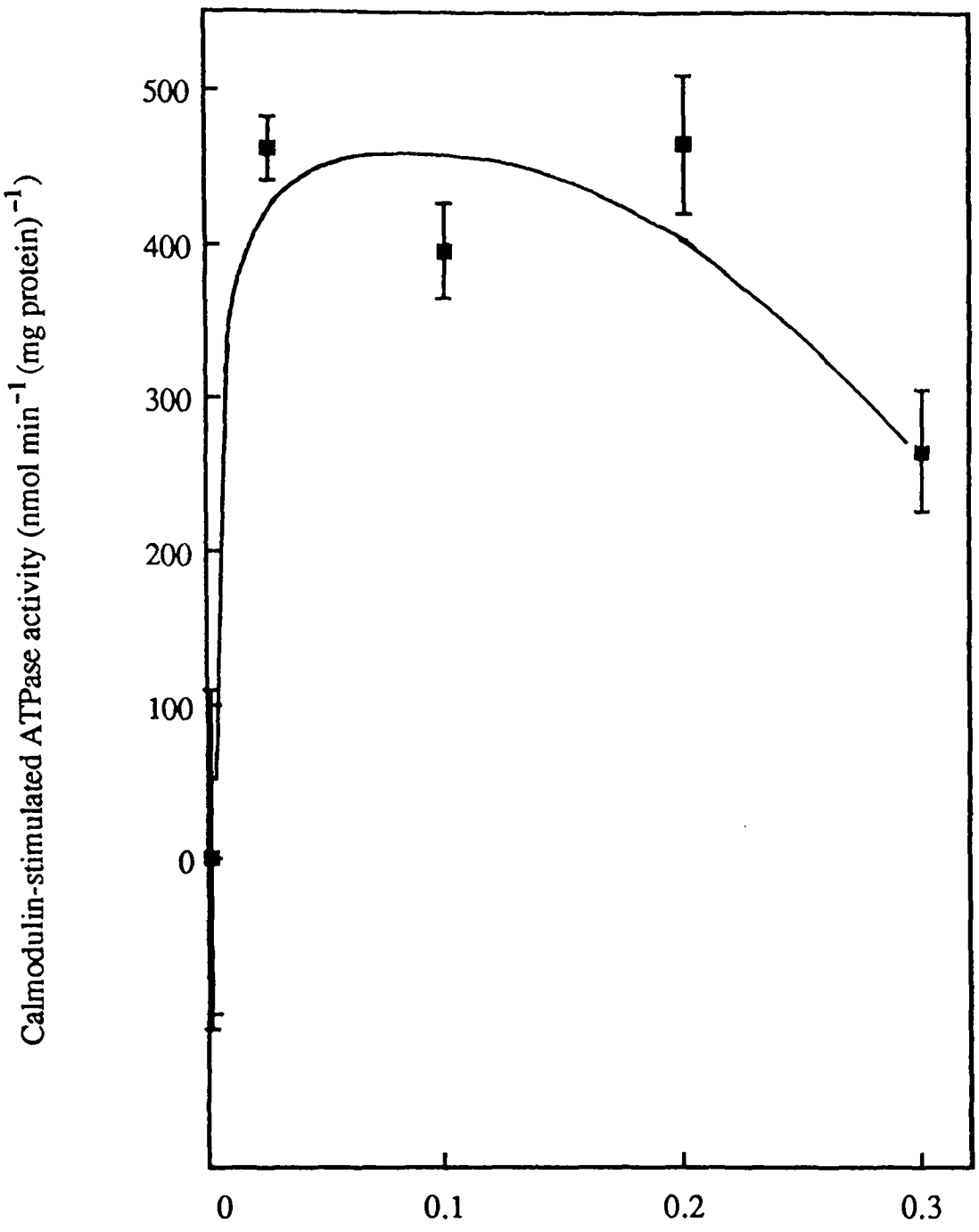

Calmodulin-stimulated ATPase activity (nmol min^{-1} (mg protein)$^{-1}$)
500
400
300
200
100
0
0
0.1
0.2
0.3
[Calmodulin] (μM)

Figure 4.9: Calmodulin-affinity chromatography of Triton X-100 solubilised microsomes; 18 hour column wash with asolectin in column buffers.

Protein content determined by absorbance at 280 nm.

Representative of three experiments.

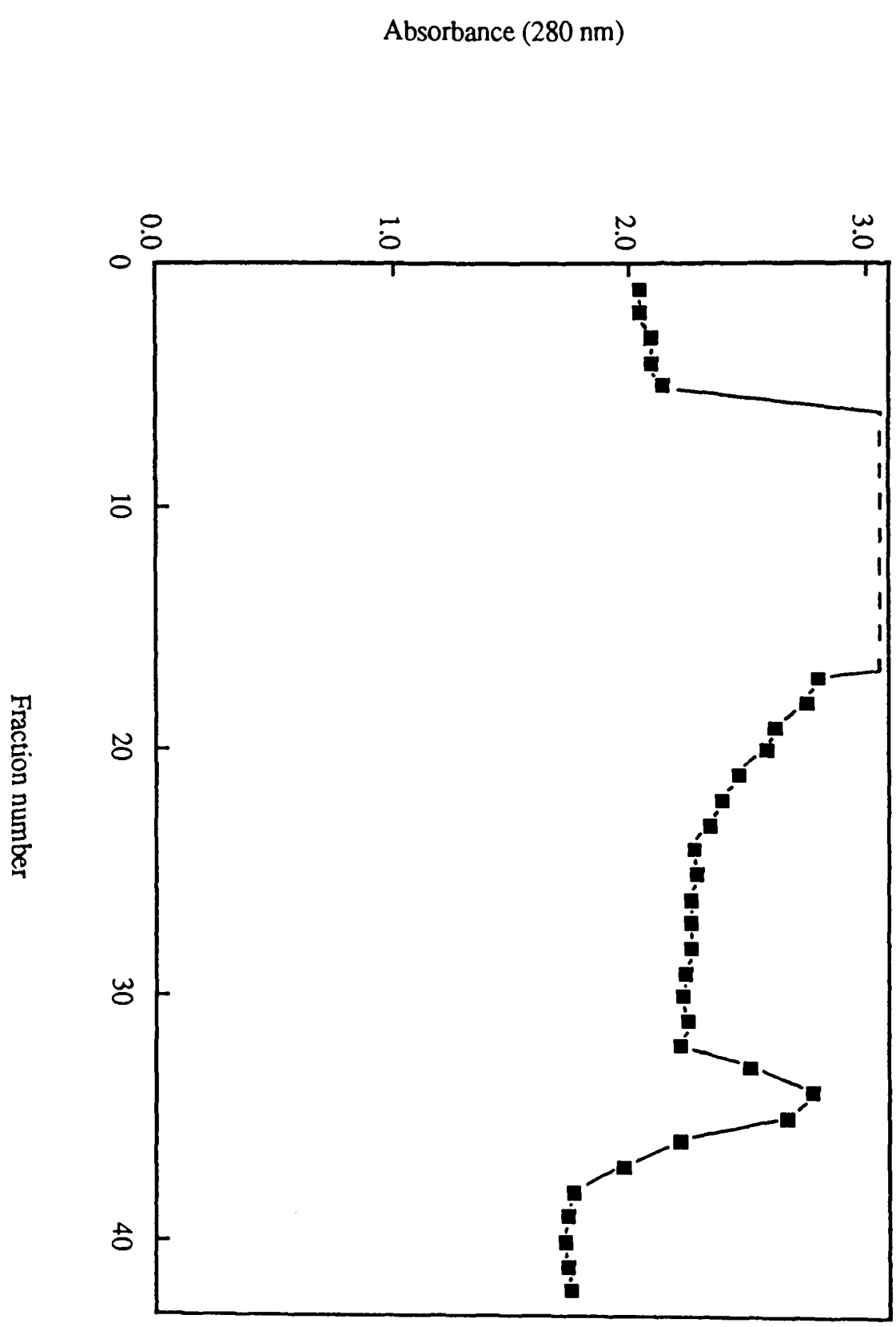
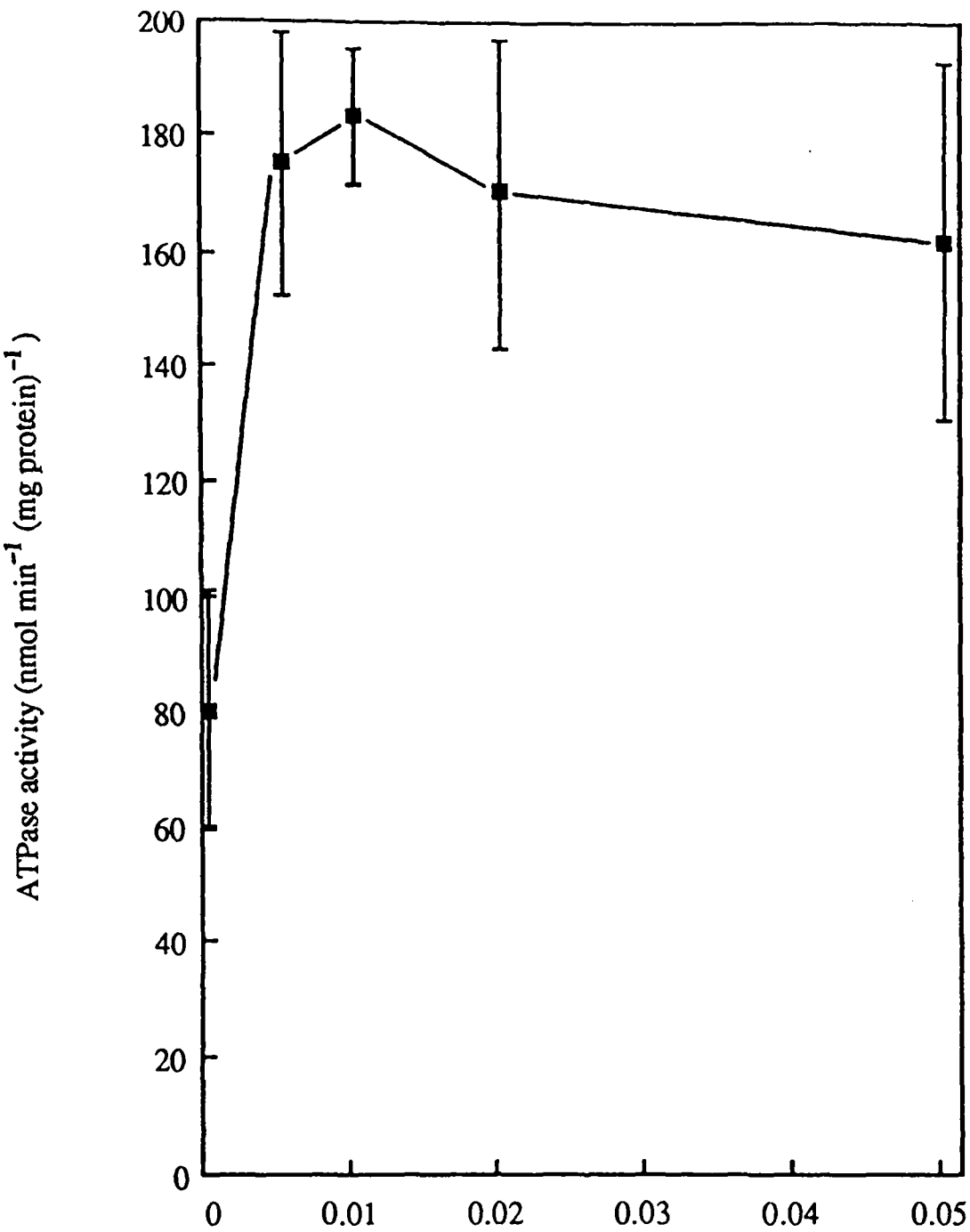

Absorbance (280 nm)
0.0
1.0
2.0
3.0
0
10
20
30
40
Fraction number

Figure 4.10: Calmodulin-stimulation of ATPase activity in EGTA-eluted peak after calmodulin-affinity chromatography (18 hour column wash with asolectin in column buffers).

Buffer background subtracted.

Representative of two experiments; values are means plus or minus one standard deviation (n = 3).


ATPase activity (nmol min^{-1} (mg protein)$^{-1}$)
200
180
160
140
120
100
80
60
40
20
0
0
0.01
0.02
0.03
0.04
0.05
[Calmodulin] (μM)

Fraction	Amount protein (mg)	ATPase activity			
		Calmodulin-stimulated		Basal	
		Total[a]	Specific[b]	Total	Specific
Homogenate	963	--	--	--	--
Microsome[c]	75	600	8	1880	25
Peak eluted after 2 hour wash	0.3	105	350	--	--
Peak eluted after 2 hour wash and reconstituted	0.1	55	500	--	--
Peak eluted after 18 hour wash with asolectin	0.09	9	100	7	80

Table 4.1: Summary of stages in purification of CSCPA using different wash conditions on the calmodulin-affinity column.

[a] Total activity units = nmol ATP hydrolysed min^{-1} (in the total fraction)

[b] Specific activity units = nmol ATP hydrolysed min^{-1} $(mg\ protein)^{-1}$

[c] Data for microsome preparation taken from radioisotope assay (see Figure 3.4)

Figure 4.11:

a. Coomassie blue stained SDS-polyacrylamide gel (7.5%) of fractions from calmodulin-affinity column (18 hour column wash, asolectin in column buffers).

Lane a: TCA-concentrated pre-peak fraction

Lane b: peak fraction

Lane c: TCA-concentrated peak fraction

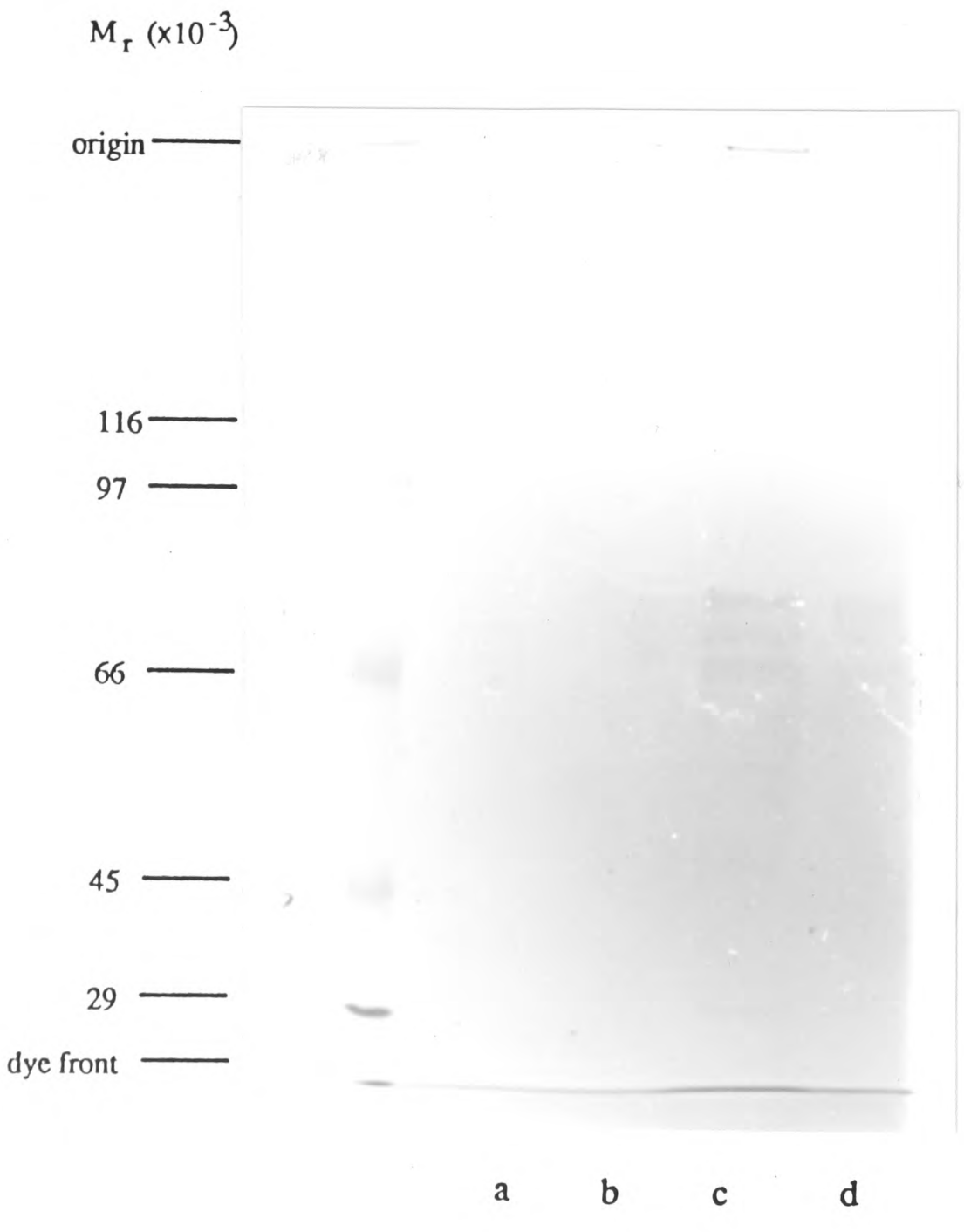
Lane d: TCA-concentrated post-peak fraction

b. Silver-stained SDS-polyacrylamide gel (7.5%) of fractions from calmodulin-affinity column (18 hour column wash, asolectin in column buffers).

Lanes a-d as above

Coomassie Blue staining was carried out by incubating the gel in 0.025% Kenacid Blue R in 50% methanol and 5% acetic acid. The gel was not fixed. Incubation was usually carried out overnight, at room temperature and with gentle agitation. Gels were destained in 5% methanol, 7.5% acetic aciduntil sufficient contrast between the bands and the background had been obtained. Silver staining was carried out as described in Figure 4.6.

a


M r (x10-3)
origin
116
97
66
45
29
dye front
a
b
c
d

b

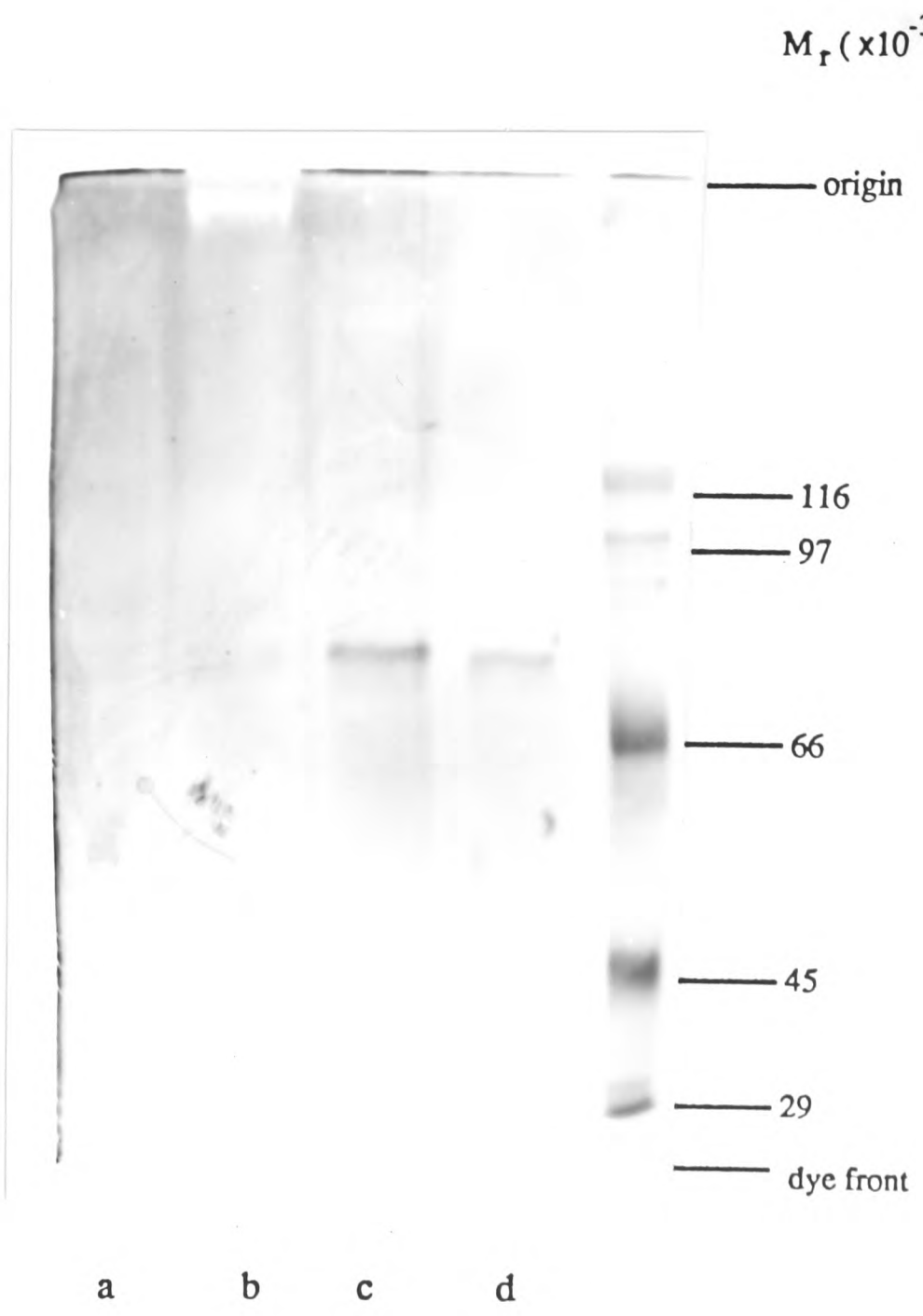

M r (x10-3)
origin
116
97
66
45
29
dye front
a
b
c
d

Figure 4.12: SDS-polyacrylamide gel electrophoresis (7.5%) of microsomes and EGTA-eluted peak from calmodulin-affinity chromatography (purification in the presence of PMSF).

Coomassie blue stained gel , followed by silver enhancement.*

Lane a: microsomes

Lane b: EGTA-eluted peak

* After staining with Coomassie Blue (see Figure 4.11), the signal was enhanced by washing in ethanol, rinsing and immersing in 0.1% silver nitrate. After washing the gel, the stain was developed by immersing the gel in 0.02% formaldehyde in 3% sodium carbonate. Staining was terminated by addition of 1% acetic acid.
This staining method was also used in Figure 4.13.

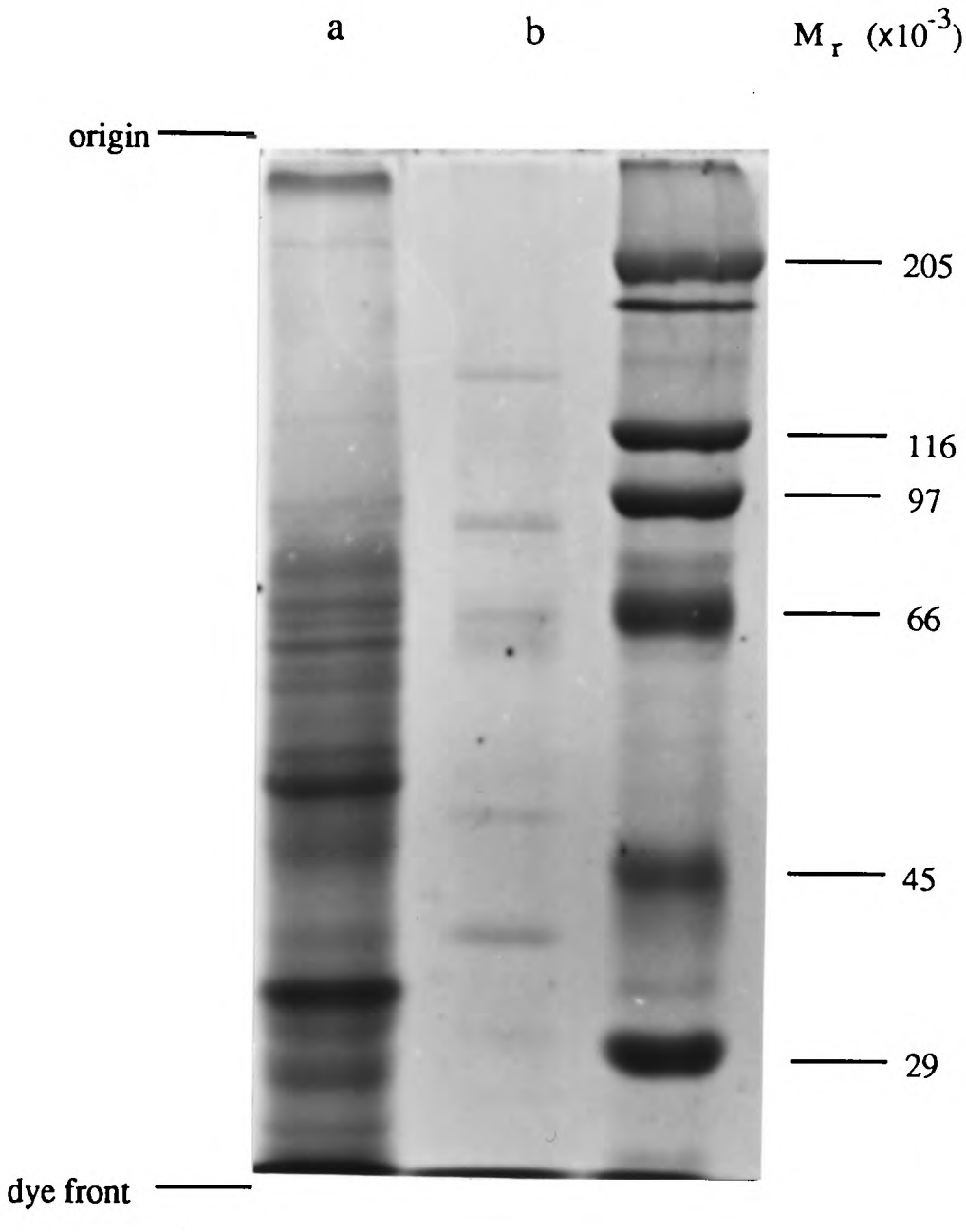

a
b
M_r ($x10^{-3}$)
origin
205
116
97
66
45
29
dye front

Figure 4.13: SDS-polyacrylamide gel electrophoresis (7.5%) of microsomes and maize CSCPA (purified in the presence of PMSF), and of erythrocyte ghosts; immunoblotting with anti-erythrocyte CSCPA antibody 97.1.

a. Maize CSCPA purification and immunoblotting.

Lane a: M_r markers, Coomassie stained

Lane b: purified CSCPA, Coomassie stained

Lane c: immunoblotting of purified CSCPA with antibody 97.1

Lane d: as lane c, without primary antibody

Lane e: microsomes, Coomassie stained

Lane f: immunoblotting of microsomes, as lane c

Lane g: microsomes as lane d

b. Erythrocyte ghost preparation.

Lane a: Aurodye stain of transferred protein

Lane b: immunoblotting with antibody 97.1

Electrophoresis and staining of gels as described in Figures 4.6 and4.11 respectively.

Proteins were electrophoretically transferred to nitrocellulose membranes by the method of Towbin *et al.*, 1979. After electrophoresis, the lane containing M_r markers was cut from the gel and stained with Coomassie Blue. The rest of the gel was sandwiched between filter paper and plastic supports, with a piece of

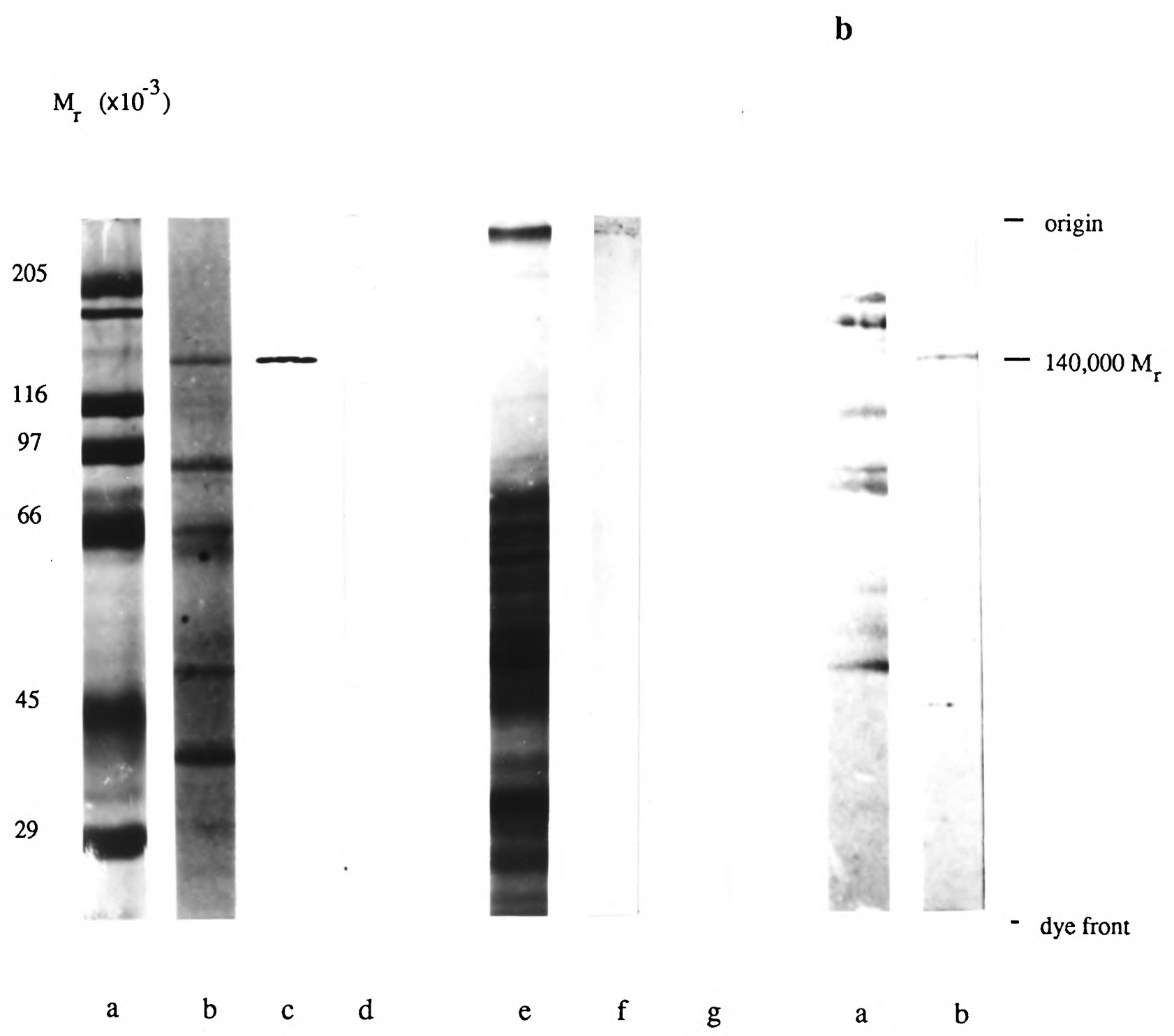
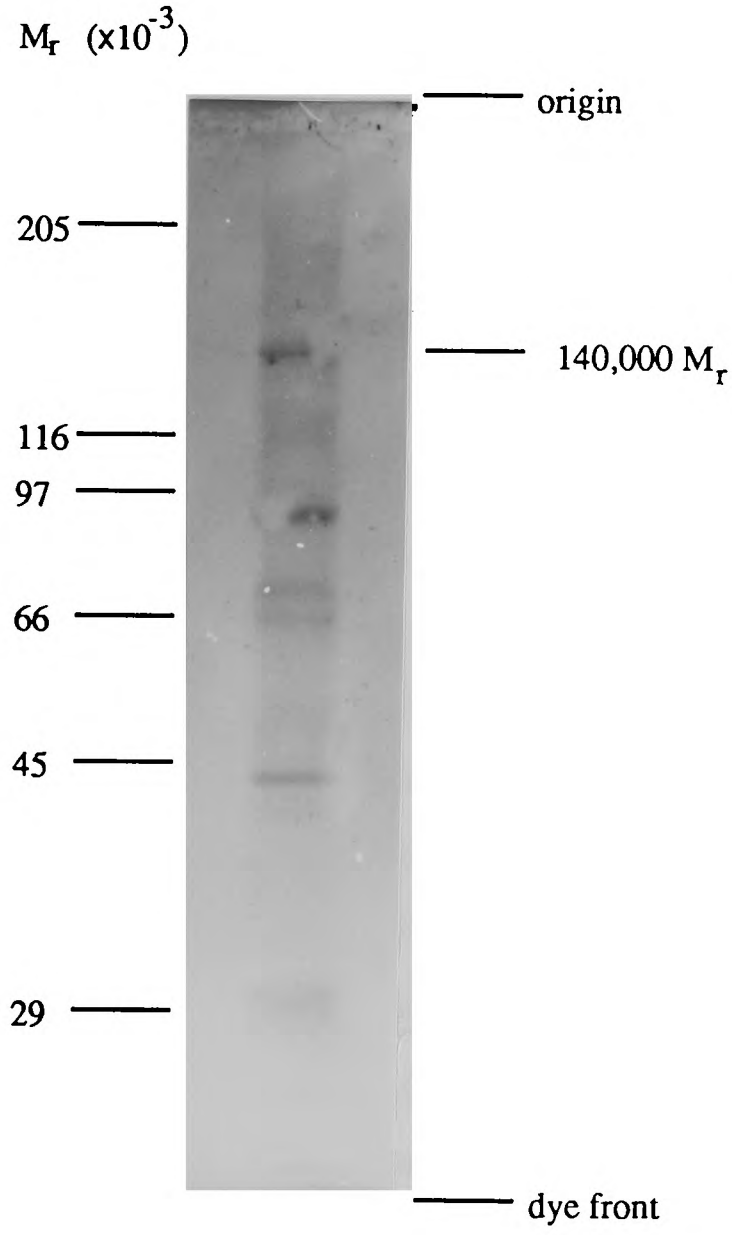

a
b
M_r ($x10^{-3}$)
205
116
97
66
45
29
origin
140,000 M_r
dye front
a
b
c
d
e
f
g
a
b

Figure 4.14: Gold-calmodulin blot of purified CSCPA electrophoretically transferred to nitrocellulose membranes from a 7.5% SDS-polyacrylamide gel.

Proteins were transferred to nitrocellulose and the membranes blocked in 0.15 M NaCl, 0.01 M Tris, 5 mg/ml gelatin, at pH 7.0. Calmodulin-binding proteins were detected using colloidal gold labelled calmodulin (100-fold dilution in the above buffer). The gold signal was enhanced with a silver enhancement kit.


M_r ($x10^{-3}$)
origin
205
140,000 M_r
116
97
66
45
29
dye front

4.3 DISCUSSION

4.3.1 Purification and reconstitution of the CSCPA by calmodulin-affinity chromatography in the absence of PMSF: assay for calmodulin-stimulated ATPase activity and SDS-PAGE of fractions

i. Purification without phospholipids.

a) Purification after a two-hour column wash.

The elution profile of the calmodulin-affinity chromatography column as measured by absorbance at 280 nm (Figure 4.1) was similar to that shown by Niggli *et al.* (1979b); Dieter and Marmé (1981b) did not present such a protein profile and their brief (2 bed volume) column wash was unlikely to have removed all the contaminant protein from the column. The data presented in Figure 4.2 for the protein content of the fractions during purification of the CSCPA indicate that 45 mg protein (the lOOKS fraction) was applied to the affinity column and that 0.3 mg was retained and eluted by EGTA. The A_{280} values of the pre- and post-peak fraction were the same as those of the buffers alone (Figure 4.1), suggesting that protein was not being eluted from the column. However, as shown in Figure 4.2, use of the protein assay of Bensadoun and Weinstein (1976) provided a more sensitive method of determining the protein content of the pre- and post-peak fractions. Results from this assay indicated that there was a small amount of protein in these fractions. This may indicate that the washing process did not remove all the unbound protein, small amounts remaining on the column and progressively washing off, both before and after elution of calmodulin-binding proteins. Alternatively, it was possible that some of the calmodulin-binding proteins eluted from the column whilst it was being washed with Buffer C, and that some of these eluted slowly after addition of Buffer D, trailing behind the main peak fraction. This latter situation has been observed by those studying the erythrocyte CSCPA (P. James, pers. comm.).

Fractions from the calmodulin-affinity chromatography column were assayed for ATPase activity, as shown in Figure 4.3. The large amount of ATPase activity in the

'non-bound' fractions may represent, *inter alia*, the activity of the proton-pumping ATPase and non-specific phosphatases. In the EGTA eluted fractions there was a small amount of ATPase activity. There was also apparent 'ATPase' activity in the pre- and post-peak fractions, but in these fractions the total activity was less than in the peak fractions. This result also indicates either that some protein containing ATPase activity was leaking slowly from the column, or that the very sensitive ATPase assay gave a false background value (see section 4:2:1 for more details of this). In the light of subsequent data, neither of these possibilities could be excluded. In the report of Niggli *et al.* (1979b) the $(Ca^{2+}+Mg^{2+})$-ATPase activity of fractions eluted from the column was shown. In this report a coupled ATPase assay was used which permitted determination of calcium- and magnesium-dependent ATPase activity. There was low $(Ca^{2+}+Mg^{2+})$-ATPase activity in the non-bound fractions, but high total activity in the EGTA eluted peak fractions. There was no $(Ca^{2+}+Mg^{2+})$-ATPase activity in the fractions before or after elution of the calmodulin-binding proteins.

When assays for calmodulin-stimulation of ATPase activity were carried out, problems were encountered in the control samples of pre- and post-peak and Buffer D, as shown in Figure 4.4a. It is not clear what the reasons were for these results, which were also found in ATPase assays of the reconstituted samples. However, in the peak fraction, but not in the controls, it was possible to demonstrate calmodulin-stimulation of ATPase activity, as shown in Figure 4.5. The data in this Figure was expressed as calmodulin-stimulated ATPase activity, for reasons described above, so it is not possible to comment on the apparent ATPase activity in the absence of calmodulin, except to point out that there was calmodulin-independent ATPase activity in the EGTA-eluted peak in the report of Dieter and Marmé (1981b). In the presence of 0.3 μM calmodulin, the specific ATPase activity was 350 nmol min^{-1} (mg protein)$^{-1}$. This result compared favourably with that of Dieter and Marmé (1981b), in which it was shown that the EGTA eluted peak contained a calmodulin-stimulated specific ATPase activity of

59 nmol min^{-1} (mg protein)$^{-1}$. In Dieter and Marmé's study, it was not made clear whether the data presented had been corrected for buffer controls. The dose-response curve for addition of calmodulin to the EGTA eluted peak (Figure 4.5) was similar to that obtained for calmodulin-stimulation of microsomal ATPase activity (Figure 3.10) and to that presented by Dieter and Marmé, in that both curves showed near maximal stimulation was reached in the presence of 0.3 μM calmodulin. In Dieter and Marmé's study, half maximal stimulation occured at about 0.15 μM, whereas in Figure 4.5 half maximal stimulation was at about 0.07 μM. The effect of calmodulin on the peak fraction indicated that a calmodulin-stimulated ATPase had been purified. The difficulties encountered in the control assays meant that it was only possible to make an estimation of the level of purification achieved on the basis of comparison of calmodulin-stimulated ATPase activity. In microsome preparations, calmodulin-stimulation of ATPase activity as measured by the radioisotope assay was about 8 nmol min^{-1} (mg protein)$^{-1}$, giving an apparent 30-fold purification. The purification achieved by Dieter and Marmé was 20-fold. These values are likely to be underestimates in both cases, since it is likely that the maize CSCPA has lost activity during purification, due either to proteolysis or removal from its membrane environment.

As indicated in the section 4:2:1 it was not possible to apply sufficient protein from samples of the column fractions to SDS polyacrylamide gels for these to be stained using a sensitive silver stain technique. When an Amicon microconcentrator was used to reduce the volume of the peak fraction to about a quarter of the original, it was necessary to centrifuge the microconcentrators for 60 minutes. After electrophoresis and silver staining of the gel, there was a strongly stained polypeptide of about 40,000 M_r (Figure 4.6) and a faintly stained polypeptide at a slightly higher M_r value. In the light of subsequent data (section 4:2:2) where it was shown that the intact maize CSCPA had a M_r of 140,000, it is possible that the polypeptides visible on this gel were proteolysis products, degradation of the CSCPA having occured during centrifugation

of the microconcentrators.

b) Purification after an 18 hour column wash.

The small amount of protein eluted by EGTA from the calmodulin-affinity column after an overnight wash (Figure 4.7) further suggested that the calmodulin-binding proteins slowly eluted from the column when calcium was present in the buffer. The lack of effect of calmodulin on the ATPase activity in the peak fraction indicated that this method, using the column buffers described, was not suitable for purification of the CSCPA. It was clear from these results that further investigations were required in order to develop purification conditions suitable for maintaining activity and integrity of the CSCPA and resolving the problems indicated with controls for the ATPase assay.

ii). Reconstitution of purified CSCPA.

The results presented in Figure 4.8 showed that it was possible to detect calmodulin-stimulated ATPase activity in the peak fraction reconstituted into asolectin vesicles. For reasons indicated previously (see section 4:2:1) the data for the peak fraction has been presented as calmodulin-stimulated ATPase activity, taking the activity in the absence of calmodulin as zero. The specific activity of calmodulin-stimulation was about 500 nmol min^{-1} (mg protein)$^{-1}$. It has been shown (Niggli *et al.* , 1981b and 1982) that there was (Ca^{2+}+Mg^{2+})-ATPase activity in the asolectin reconstituted erythrocyte CSCPA; the total activity was in the region of 0.9 nmol min^{-1}. It has been stated by Carafoli *et al.* (1980a) that 'the ATPase reconstituted into asolectin displayed a lack of responsiveness to calmodulin' although the data was not published. Carafoli *et al.* (1980a) suggested that acidic phospholipids activated the ATPase and substituted for the effect of calmodulin. It is not known at present whether such phospholipid regulation of ATPase activity of the maize CSCPA occurs. The results presented in Figure 4.8 could be taken to suggest that phospholipid regulation of the maize CSCPA is different from that of the erythrocyte CSCPA. However, this cannot be stated with certainty from these preliminary experiments since the composition of the asolectin used was not

known in detail. It is also suggested from this data that the ATPase was activated by low calmodulin concentrations, maximal activation occuring at about 0.025 μM calmodulin. This was lower than that found in the assays for calmodulin-stimulation of microsomal ATPase activity (maximal at 0.05-0.1 μM calmodulin, according to the assay method) and lower than in the CSCPA purified without reconstitution (maximal at upward of 0.3 μM calmodulin). The lower amount of calmodulin required for activation of the asolectin reconstituted CSCPA is likely to be a result of the fact that the effect of calmodulin is a function of the amount of calmodulin-binding protein present in the assay as well as calmodulin concentration. Alternatively, it may be suggested that the affinity of the calmodulin-binding domain of the CSCPA for calmodulin was greater after reconstitution. It may also be that some of the phospholipids contained in asolectin interacted in some way with the CSCPA after reconstitution such that its sensitivity to calmodulin was enhanced. From this data the possibility that an enzyme had been co-purified which was not the CSCPA, and which had a different calmodulin dose-response curve, could not be excluded. However, this seems unlikely in the light of subsequent data.

iii). Purification with asolectin in the column buffers.

The column profile at 280 nm for purification of the CSCPA by this method showed an apparently larger peak fraction (Figure 4.9) than that obtained after an overnight wash without asolectin (Figure 4.7). However the amount of protein measured in the peak after an overnight wash with asolectin containing buffers was the same as that after the overnight wash without asolectin (0.09 mg with asolectin, 0.09 mg without asolectin). This indicates that absorbance at 280 nm was not a reliable method with which to compare the amount of protein eluted from an affinity column, especially if Triton X-100 and phospholipid were present in the column buffers. The results obtained from the protein assay indicated that there was an amount of protein which remained firmly bound to the column after an overnight wash, the calmodulin-stimulated ATPase

activity of which was entirely dependent on the presence of asolectin. The results obtained for purification of the CSCPA by this method were improved in that the control values were lower than those of the peak sample and correction for these could be made in the calculation of specific activity (Figure 4.10). The calmodulin-stimulated component of ATPase activity was about 100 nmol min^{-1} (mg protein)$^{-1}$, which was lower than that obtained after a two hour column wash without asolectin and lower than that in the asolectin reconstituted sample. There was some basal calmodulin-independent ATPase activity in the peak purified by this method. This may suggest that the CSCPA had a basal calmodulin-independent ATPase activity, as is the case for the erythrocyte CSCPA. In this sample, as for the CSCPA reconstituted into asolectin, the amount of calmodulin required for maximal stimulation was lower than that for the CSCPA purified without phospholipids. This may lend further weight to the suggestions made above concerning the effect of asolectin on the CSCPA. However, given the problems indicated above in the buffer controls for assays of ATPase activity after a two hour column wash and after reconstitution into asolectin, it is difficult to assess the importance of the differing levels of calmodulin-stimulation. The results obtained for assays of calmodulin-stimulated ATPase activity are shown in Table 4.1 for comparative purposes.

It was shown (Figure 4.11) that TCA-precipitation was a suitable method for concentrating the peak, pre-peak and post-peak samples eluted from the calmodulin-affinity column prior to electrophoresis (compare lanes b and c of Figure 4.11a). In the TCA precipitated peak, three polypeptides were visible, of M_r 62,000, 66,000 and 72,000. The lack of staining of the 62,000 and 66,000 M_r polypeptides by the silver staining technique may have arisen as a consequence of the different amino acid residues to which the stains bind. The three polypeptides visible in the Coomassie Blue stained gel were of a smaller size than reported for the intact erythrocyte CSCPA, and may represent proteolysis products of the intact maize CSCPA (see section 4:3:2 below). If it is assumed that degradation of the polypeptides did not occur during electrophoresis, then it

appears that polypeptides of M_r 62,000-72,000 were functional in terms of calmodulin-stimulation of ATPase activity. It has been shown from studies of trypsin digestion of the purified erythrocyte CSCPA that a proteolysis product of approximately 90,000 M_r contains the domains necessary for calmodulin-stimulated ATPase activity (Graf *et al.* , 1982; Carafoli *et al.* , 1982; Zurini *et al.* , 1984; Benhaim *et al.* , 1986). A proteolysis product of the erythrocyte CSCPA of about 76,000-81,000 M_r was shown to be active with respect to ATPase activity, but the effect of proteolysis was to activate the ATPase activity such that it was no longer sensitive to calmodulin. It is possible that the maize CSCPA may have a slightly different proteolysis pattern to the erythrocyte CSCPA in terms of functional subunits, an issue discussed further in section 4:3:2 and in Chapter 7.

There was evidence from these gels that the calmodulin-affinity column did not achieve a perfect separation, with low levels of protein released before and after the EGTA wash, as shown by the presence of polypeptides in the TCA-concentrated pre- and post-peak fractions (Figure 4.13a, lanes a and d; Figure 4.13b, lane d). Possible reasons for this have been discussed above. In view of the fact that Coomassie Blue appeared to give a satisfactory result in staining the peak fraction it was decided to use this stain in subsequent gels rather than the silver stain of Morrissey (1976). In some experiments it was necessary to increase the contrast of the Coomassie Blue stained bands by means of the silver enhancement technique of Gorg *et al.* (1975).

4.3.2 Purification of the maize CSCPA in the presence of PMSF: SDS-PAGE, immunoblotting and detection of calmodulin binding proteins

i). SDS-PAGE.

The results shown in Figure 4.12 (lane b) for electrophoresis of the EGTA eluted peak

fraction (after purification in the presence of PMSF and concentration by TCA precipitation) indicated the presence of a polypeptide of M_r 140,000 (±10,000 M_r). This value is close to that obtained in the later determinations of M_r for the erythrocyte CSCPA; a value of 138,000 ±4,000 M_r was reported by Graf *et al.* (1982), which was slightly higher than that originally estimated by Niggli *et al.* (1979b) and was suggested to be more accurate as a result of a better choice of electrophoresis media. The calculated M_r of the erythrocyte CSCPA from the amino acid sequence deduced from the cDNA sequence was 134,683 (Verma *et al.* , 1988). The similarity in M_r between the polypeptide purified by calmodulin-affinity chromatography from maize and the intact erythrocyte CSCPA suggests that the maize CSCPA may be similar to the erythrocyte one. In the purification of the erythrocyte CSCPA by calmodulin-affinity chromatography (Niggli *et al.* , 1979b) the Triton X-100 solubilised fraction was concentrated using a 100,000 M_r cut-off membrane. It is therefore not surprising that the gels of the erythrocyte CSCPA showed only the band at 140,000 M_r and none below 100,000 M_r. In Figure 4.12, lane b it can be seen that there were also a series of other polypeptides of lower M_r in the peak sample from maize. It is interesting to note that the M_r values of these polypeptides (91,000, 77,000-69,000, 51,000 and 40,000) were similar to the M_r values of the major products from limited trypsin digestion of the purified erythrocyte CSCPA (of M_r values 90,000, 81,000-76,000, 50,000 and 42,000; Graf *et al.* , 1982; Carafoli and Zurini, 1982). This raised the possibility that the polypeptides of less than 140,000 M_r seen in the maize preparation were proteolysis products of the intact CSCPA. It is known that higher plants contain proteinases with properties similar to serine proteases found in animal systems. For example, Shannon and Wallace (1979) demonstrated that maize roots contained a serine protease-like activity; Ryan and Walker-Simmons (1981) have reviewed the evidence for proteinases in plants. However, in the light of the information presented in this chapter, the possibility that these may be other calmodulin-binding proteins cannot at present be excluded (but see evidence from monoclonal antibodies

presented in Chapter 6). The results presented in Figure 4.12 indicated the importance of the buffer and purification conditions used in the preparation of integral membrane proteins. The way in which samples of the peak proteins from the calmodulin affinity column were prepared for electrophoresis had important consequences in the pattern of polypeptides that were seen. In this study, the important stages in the preparation of samples of the CSCPA for electrophoresis were the presence of protease inhibitors in the sample buffer for electrophoresis, incubating the samples on ice in electrophoresis buffer (rather than at 100°C) and the use of 'Eppendorf' tubes coated with 'Repel-Silane'. It is interesting to note for comparison that the erythrocyte CSCPA preparation of Niggli *et al.* (1979b) was incubated at 100°C prior to electrophoresis, after which the 140,000 M_r polypeptide was visible.

ii). Immunoblotting.

Confirmation that the 140,000 M_r polypeptide was the maize CSCPA was provided by the experiment shown in Figure 4.13a, which demonstrated that the 140,000 M_r polypeptide showed antigenic homology with the erythrocyte CSCPA. The polyclonal antibody 97.1 raised to the CSCPA from human erythrocytes showed strong cross reaction at 140,000 M_r with the fraction produced by calmodulin-affinity chromatography from maize coleoptiles. This indicated that there was antigenic homology between the two calcium transporters and provided good evidence bearing out the suggestion of Dieter and Marmé (1981b) that microsomal plant membrane fractions contain a calmodulin-stimulated, calcium pumping ATPase similar to that in erythrocytes.

Antibody 97.1 was a monospecific, affinity-purified, polyclonal antibody showing identical properties to those described by Verma *et al.* (1982), (F. Kessler, pers. comm). It was shown that these antibodies did not affect binding of calmodulin to the erythrocyte membranes, and it was suggested that the antibodies were not recognising the calmodulin-binding domain of the eryhtrocyte CSCPA. The suggestion was made by

Verma *et al.* (1982) that 'antibodies were raised against the portions of the Ca^{2+}-ATPase structure which are involved in the expression of the enzyme activity and calcium transport'; this was made on the basis of the effect of the polyclonal antibodies on Ca^{2+}-ATPase activity and calcium transport in inside out erythrocyte ghost vesicles. For both Ca^{2+}-ATPase activity and calcium transport it was shown that the antibodies inhibited activity in the presence and absence of calmodulin, although the data was not presented in a form which made it possible to determine whether the effect was on calmodulin-stimulated activity or on basal activity alone. As antibody 97.1 had the same properties as the polyclonal antibodies described by Verma *et al.* (1982) it may be suggested that 97.1 recognised a homologous conserved region found in the erythrocyte and maize CSCPA which was possibly at or near the active site. Verma *et al.* (1982) demonstrated that anti-human erythrocyte Ca^{2+}-ATPase serum showed a lack of cross reactivity with pure rabbit skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase, which is not stimulated by calmodulin. Cross reactivity between anti-human erythrocyte CSCPA serum and Triton X-100 extracts of erythrocyte ghosts from other species was demonstrated. Further evidence concerning the lack of overall similarity between sarcoplasmic reticulum-type and plasma membrane-type calcium transporters has been obtained from cDNA sequences. Comparison of the deduced amino acid sequence of rabbit fast twitch skeletal sarcoplasmic reticulum Ca^{2+}-ATPase (MacLennan *et al.* , 1985) and the erythrocyte CSCPA (Verma *et al.* 1988) showed that there is little overall sequence identity between these proteins, with the exception of regions conserved in all ion transporters sequenced to date. These regions are those surrounding the phosphorylation site, the ATP binding site and the 'hinge' region. The hinge region is thought to permit intramolecular movements necessary to bring spatially separate catalytic sites together, a process essential for some of the energy transduction features of P-type ion transporters (Verma *et al.* , 1988). The binding of antibody 97.1 (which is specific to a plasma membrane component in erythrocytes) to the maize CSCPA suggests that it

also is present in the plasma membrane (see Chapter 7 for general discussion).

The cross reaction of antibody 97.1 with a polypeptide at 140,000 M_r from erythrocyte ghosts indicated that 97.1 was behaving as expected (Figure 4.13b, lane b). Antibody 97.1 did not bind detectably to any polypeptides in the maize microsome fraction, as shown in Figure 4.13a, lane f. The lack of detectable binding may be a result of the lower amount of CSCPA present in the mixture of proteins applied to the gel. Cross reaction with erythrocyte ghost proteins may be explained by the larger amount of CSCPA present amongst the membrane proteins. Another possibility was that the binding of antibody 97.1 was dependent on the conformation of the CSCPA, in such a way that it was possible in the purified maize CSCPA but not in the microsomal fraction. It may also be that binding was affected by the presence of membrane phospholipids in the maize microsome sample.

iii). Detection of calmodulin-binding to purified CSCPA.

Figure 4.14 shows that gold-calmodulin bound to the intact maize CSCPA at 140,000 M_r after purification in the presence of PMSF as described in section 4:2:2 above. The gold-calmodulin also bound to other polypeptides in the purified CSCPA fraction, at 91,000, 77,000-69,000, 51,000 and 40,000 M_r. If these polypeptides were proteolysis products of the 140,000 M_r CSCPA, then it may be suggested that the calmodulin-binding domain of the maize CSCPA was contained in all these proteolysis products. An alternative explanation is that these lower M_r bands are not related to the CSCPA, but are other calmodulin-binding proteins which have been purified with the CSCPA. In the studies on trypsin degradation of the intact erythrocyte CSCPA it was shown that only some of the proteolysis products were capable of binding calmodulin. The experiments were carried out by means of incubating the purified CSCPA (after exposure to trypsin for the required length of time) with ^{125}I-labelled azido-modified calmodulin (Carafoli *et al.* , 1982). The samples were then electrophoresed and it was shown that the calmodulin-binding domain was present in the intact CSCPA, in the 90,000 M_r

proteolysis product and in a limit polypeptide of about 25,000 M_r which occured after prolonged exposure to trypsin. Reasons for this apparent discrepancy in the results may lie in the presence of different proteolytic pathways in the tissues or in the presence of other calmodulin binding proteins in the maize preparation.

From the data presented in this chapter, it may be suggested that the intact maize CSCPA undergoes proteolysis to form a variety of polypeptides. After purification in the presence of asolectin in the column buffers, it was shown that three polypeptides of M_r 62,000, 66,000 and 72,000 were present (Figure 4.13, lane c) and calmodulin-stimulated ATPase activity was shown in this fraction. Therefore, if degradation of the polypeptides did not take place during electrophoresis, it may be that these 62,000-72,000 M_r polypeptides contained both the active site and the calmodulin-binding domain of the CSCPA. Further evidence which lends weight to the suggestion that polypeptides in the region of 60,000-70,000 M_r (and possibly at 50,000 M_r also) were proteolysis products of the intact CSCPA may be found in Chapter 6. It may be that there is more than one pathway of proteolysis (as has been suggested for the erythrocyte CSCPA; Carafoli and Zurini, 1982); if so, the polypeptides at 62,000, 66,000 and 72,000 M_r observed after purification in the presence of asolectin may be derived from a different pathway to those at M_r 77,000-69,000 observed after purification in the presence of PMSF. It is likely that the polypeptide at 40,000 M_r observed after microconcentration and after purification with PMSF is a limit polypeptide of one of the proteolytic pathways.

The results obtained in collaboration with Professor E. Carafoli, Eidgenössiche Technische Höchschule, Zürich, concerning the amino acid sequence of limited regions of the maize CSCPA (see Appendix II) were of importance in providing further evidence concerning the similarity of the maize CSCPA to the erythrocyte CSCPA. The plant peptide CNBr1 (GIAGSDAAR) showed strong similarity (80%) with the 'hinge' region (GIAGTDVAK) of the erythrocyte calmodulin-dependent Ca^{2+}-ATPase. This plant peptide was also similar (77.8%) to the 'hinge' sequence (GIAGSDVSK) of

the Na^+/K^+-transporting ATPase from HeLa cells. The homology (80%)shown by the maize peptide CNBr2 (THPEFLLDQD) to the erythrocyte calmodulin-dependent Ca^{2+}-ATPase sequence (THPEFRIEDS) was important because this sequence is located in the C-terminal extension found only in calmodulin-stimulated, calcium transporting ATPases. The peptide CNBr2 overlaps the beginning of a region bearing a strong negative charge. This region begins at the sequence IEDS; the corresponding sequence in the plant peptide is LDQD which contains three negatively charged amino acid residues. Therefore it is possible that the maize CSCPA also has negatively charged domains. The amino acids contained in the C-terminal extension of the erythrocyte CSCPA are involved in calmodulin-binding and calcium-binding. There is also a sequence which is phosphorylated in a cyclic AMP-dependent manner, which regulates activity (Verma *et al.* , 1988). No evidence as yet is available concerning whether such a sequence exists in the maize CSCPA; the presence of such a region (if functional)would be of great interest since, although the presence of cAMP has been shown in plant extracts, no reliable reports exist as to its physiological role (see Chapter 1). This amino acid sequence data provided the basis on which to progress further and determine the cDNA sequence of the maize CSCPA in order to examine the level of homology between this and other ion-translocating ATPases. Such work is currently being carried out by Dr. D.E. Evans (Plant Sciences, Oxford) and P. James (Eidgenössiche Technische Höchschule, Zürich).

Chapter 5

INVESTIGATIONS OF THE REACTION MECHANISM OF THE MAIZE CALMODULIN-STIMULATED ATPase

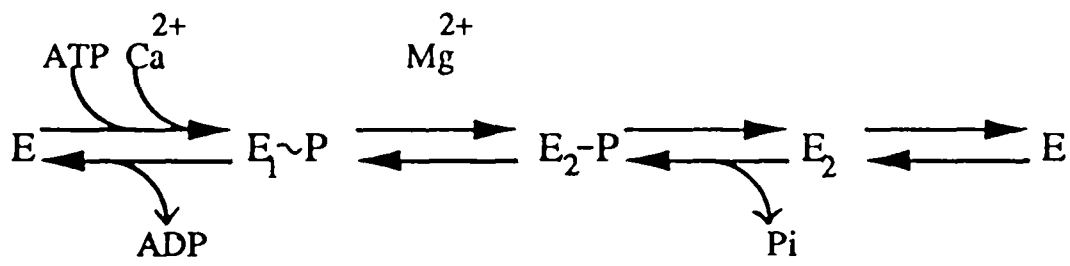
5.1 INTRODUCTION

Evidence presented in Chapter 3 and Appendix I indicated the presence of a calmodulin-stimulated, calcium pumping ATPase (CSCPA) in microsomal fractions from maize coleoptiles. The CSCPA was purified for the first time in its intact form, and shows homology and similar properties to the erythrocyte calmodulin-stimulated, calcium-pumping ATPase (Chapter 4). In the light of these results, a further study of the maize CSCPA was carried out to investigate whether phosphorylated intermediate formation occured as a part of its reaction mechanism in a similar manner to other ion translocating

ATPases.

5.1.1 Reaction mechanism of P-type ATPases

An essential feature of P-type ion transport ATPases is that the γ-terminal phosphate group from ATP forms a covalent link with a single aspartyl residue located in a specific phosphorylation domain of the polypeptide (Pederson and Carafoli, 1987a and 1987b). Subsequently, a conformational change is initiated in regions of the polypeptide distinct from the phosphorylation site, such that ions are translocated from high affinity binding sites on the cytosolic face to low affinity sites on the other side of the membrane. In this way, directional movement of ions is brought about, coupled to the use of metabolic energy. Therefore, P-type ion translocating ATPases exist in two conformational states, which form the basis of the reaction mechanism. A simplified reaction scheme is illustrated below:



(Muallem and Karlish, 1980)

Investigation of the formation of phosphorylated intermediates can be carried out in appropriate reaction conditions. The phosphorylated intermediate is formed very rapidly and is highly sensitive to temperature and alkaline conditions. Methods were originally developed in the study of mammalian Na^+/K^+ ATPases (Post *et al.* , 1965;

Bader and Post, 1966; Post and Sen, 1967). Essentially, these methods involve incubation of a sample of ATPase in a medium containing ATP labelled in the γ-position with ^{32}P, for short periods (usually a few seconds) and at low temperatures. The reaction is terminated by addition of an acid solution to precipitate phosphorylated protein and remove unincorporated label. Incorporation of a label may be detected by scintillation counting, or by gel electrophoresis under acid conditions followed by autoradiography which permits determination of the M_r of phosphorylated peptides. These experimental conditions minimise phosphorylation arising from the activity of most protein kinases. In the studies described below, these basic principles were employed, with minor modifications.

5.1.2 Features of phosphorylated intermediate formation by calcium-pumping ATPases in erythrocyte membranes

This topic has been reviewed recently by Rega and Garrahan (1986). The first demonstration of calcium stimulated phosphoprotein formation in human red blood cell ghosts was made by Knauf *et al.* (1974), who presented evidence for a calcium-stimulated phosphoprotein in red blood cell ghosts that was distinct from the sodium-stimulated phosphoprotein. Formation of the calcium-stimulated phosphoprotein was dependent on the presence of magnesium. The M_r of the calcium-stimulated phosphoprotein was estimated as 150,000, although there was a fainter band running at approximately 142,000 M_r (see section 5:3 for further discussion of this). Formation of the phosphorylated intermediate was shown to be calcium-dependent (Rega and Garrahan, 1975) although this result was not found in all the early experiments on red cell ghost phosphorylation (see section 5:3). The formation of phosphorylated intermediates in red blood cell membranes has been shown to be diminished after isotopic dilution of the [$\gamma^{32}P$]-ATP (i.e. adding a 1 minute chase of unlabelled ATP in excess over the radiolabel added initially) (Katz and Blostein, 1975; Szasz *et al.* , 1978). Identification of the type of amino acid

to which the γ-phosphate binds has been made following treatment of the phosphorylated intermediate with hydroxlamine. The effect of hydroxylamine is to specifically cleave acyl-phosphate bonds (Lipmann and Tuttle, 1945). In the study by Rega and Garrahan (1975) red blood cell membranes were phosphorylated and the pellet of precipitated protein suspended in a hydroxylamine solution. After this treatment, ^{32}P was released from the membranes, indicating the formation of an acyl-phosphate. This acyl-phosphate was sensitive to pH, maximal stability of the phosphorylated protein being at pH 1. Inclusion of lanthanum in the medium for phosphorylation brought about an increase in the amount of phosphorylation detected, both for total membrane phosphorylation and for phosphorylation of the 150,000 M_r polypeptide (which was suggested to represent the calcium pump, Szasz *et al.* , 1978). Lanthanum is known to enhance the apparent extent of phosphorylation by means of inhibiting the dephosphorylation process (Luterbacher and Schatzmann, 1983). It was proposed that lanthanum blocks the transition between E_1~P and E_2-P. Inclusion of calmodulin in the reaction medium (Muallem and Karlish, 1980) enhanced the rate of calcium-dependent phosphorylation at least ten-fold. It was suggested that the conformational equilibrium between the E_1 and E_2 states of the enzyme was effected by calmodulin.

5.1.3 Features of phosphorylated intermediate formation by the purified erythrocyte calmodulin-stimulated, calcium pumping ATPase

Evidence concerning the formation of phosphorylated intermediates in partially purified erythrocyte CSCPA was presented by Lichtner and Wolf (1980a and b). Niggli *et al.* (1979b) purified the CSCPA to near homogeneity (see Chapter 4, section 4:1) and demonstrated that the CSCPA formed a phosphorylated intermediate of M_r approximately 125,000, which was formed only in the presence of calcium. Subsequently, several authors (for example, Stieger and Luterbacher, 1981; Allen *et al.* , 1987) have

demonstrated that purified CSCPA forms a phosphorylated intermediate of equivalent properties to those indicated above for the membrane-bound CSCPA. These studies have enabled further details of the reaction mechanism of the CSCPA to be proposed (reviewed by Rega and Garrahan, 1986). The discrepancies in the estimations of M_r for the phosphorylated intermediates of the erythrocyte CSCPA are likely to be a feature of the different gel systems used.

5.1.4 Studies on phosphorylated intermediate formation by P-type ATPases in higher plants

As described in Chapter 1, current evidence concerning P-type ion-translocating ATPases in higher plants has suggested that these fall into several categories, as determined by the ion translocated and by subcellular location. Of these, the proton-pumping ATPase of the plasma membrane has been the most extensively investigated (for general reviews see Sze, 1985; Serrano, 1988 and 1989). Briskin and Leonard (1982a and b) were the first to report that a phosphorylated intermediate was formed in corn root plasma membrane fractions. The M_r of this phosphoprotein was estimated at 100,000, and its properties were shown to correlate with those of H^+-ATPase activity. Scalla *et al.* (1983) demonstrated that formation of the maize root phosphorylated intermediate at 100,000 M_r was dependent on the concentration of ATP and was inhibited by vanadate and diethylstilbestrol. Other species were studied by Vara and Serrano (1983) who showed phosphorylated intermediate formation in the purified plasma membrane ATPase of *Avena sativa* L. The work of Briskin and co-workers on *Beta vulgaris* L. enabled further details of the reaction mechanism of the H^+-ATPase to be elucidated (Briskin and Poole 1983a and b; Briskin and Thornley, 1985; Briskin, 1986; Briskin 1988a and b).

The endoplasmic reticulum calcium pump is another P-type ATPase that has been characterised by formation of phosphorylated intermediates (Giannini *et al.* , 1987a).

Purified endoplasmic reticulum vesicles were incubated with [γ^{32}P]-ATP, and the phosphorylated intermediate formed had a M_r of 96,000. Phosphorylation of this polypeptide was reduced after isotopic dilution of the [γ^{32}P]-ATP, although under the conditions used (20 seconds phosphorylation with [γ^{32}P]-ATP, followed by 40 seconds with a 100-fold excess of unlabelled ATP) some phosphorylation was still present. Treatment with hydroxylamine also diminished phosphorylation, but again incompletely. No data were presented relating to the calcium-dependence of phosphorylated intermediate formation, although both ATPase and calcium transport activities were dependent on the concentration of calcium. The plant endoplasmic reticulum calcium pumping-ATPase appears to be insensitive to calmodulin (see Chapter 1).

The results presented in this chapter are the first to describe the properties of phosphorylated intermediate formation by a higher plant calmodulin-stimulated, calcium pumping ATPase, and permit further comparison to be made between P-type ATPases (Chapter 7). Results of this work have been presented by Briars and Evans (1989).

5.2 RESULTS

Formation of phosphorylated intermediates by the calmodulin-stimulated, calcium pumping ATPase of maize coleoptiles was investigated using the protocol described in Chapter 2, section 2:2:7. PMSF was included in the preparation of microsomes and purified CSCPA, allowing phosphorylation of the intact CSCPA to be studied; samples were prepared for acid gel electrophoresis without boiling. Within each experiment, care was taken to ensure that the protein content of assays and of samples loaded onto gels was equivalent, such that visual comparisons could be made between the intensity of phosphorylated bands in different lanes of the same gel. For each experiment relative molecular mass markers were run on the gels; representative graphs of log M_r against R_f have been presented for Weber and Osborne gels at pH 6.0 (Chapter 2, section 2:3:6). Such a plot was constructed for each experiment, and used in the estimation of M_r. To permit reliable estimation of M_r values of phosphorylated bands to be made, experiments were repeated at least twice using 5% and 7.5% acid gels. After estimation of M_r values had been made for each experiment, the results were averaged, and it is these average values of M_r for the various bands that are discussed below. Where appropriate, results from representative 5% and 7.5% gels are presented in this chapter; the M_r values are to $\pm 7\%$ or less.

5.2.1 Phosphorylation in the presence of calcium

A band of phosphorylation at M_r 140,000 was present in most of the microsome preparations incubated in the presence of calcium, with 1 μCi [γ^{32}P]-ATP (Figure 5.1, lane b; Figure 5.2, lane b; Figure 5.9, lane a; Figure 5.10, lane a). The band of phosphorylation at 140,000 M_r was also apparent after incubation in the presence of 5 μCi [γ^{32}P]-ATP with 2 μM unlabelled ATP (Figure 5.3, lane b; Figure 5.6, lane a). The free calcium concentration in these assays was estimated as 5×10^{-5} M using a calcium-sensitive electrode.

These bands of phosphorylation were apparent after only 24 hours autoradiography,

although comparison indicates that the extent of phosphorylation was not directly comparable between different gels. Phosphorylation was enzymic in nature, as shown by the complete absence of phosphorylation in microsome samples that had been boiled for 2 minutes prior to incubation with [γ^{32}P]-ATP (Figure 5.11, lanes g and h).

The purified CSCPA also showed a band of phosphorylation in the presence of calcium at 140,000 M_r (Figure 5.4, lane b; Figure 5.5, lane b) in the presence of labelled ATP. In these assays, free calcium was in the region of $7x10^{-6}$ M. This band was also apparent after incubation in the presence of labelled and unlabelled ATP (Figure 5.7, lane b).

After phosphorylation of microsome preparations in the presence of 50 μM calcium followed by isotopic dilution of the ATP, the 140,000 M_r phosphorylated band was absent (with 1 μCi [γ^{32}P]-ATP, Figure 5.1, lane g; Figure 5.2, lane g: with labelled and unlabelled ATP, Figure 5.3, lane h; Figure 5.6, lane h). The same result was shown for purified CSCPA (1 μCi ATP, Figure 5.4, lane g; Figure 5.5, lane g: 2 μM plus 5 μCi ATP, Figure 5.7, lane e).

After termination of the reaction in the presence of calcium, samples of microsomes and purified CSCPA were washed with hydroxylamine solution. This treatment caused the phosphorylated band at 140,000 M_r to be abolished, as shown for microsome preparations (Figure 5.1, lane h; Figure 5.2, lane h; Figure 5.3, lane f; Figure 5.6, lane e). Control treatment (washing microsome pellets in MES-KOH only) did not reduce phosphorylation (Figure 5.6, lane f compared with lane a). Phosphorylation at M_r 140,000 in the purified CSCPA preparation was also abolished by hydroxylamine (Figure 5.4, lane h; Figure 5.5, lane h; Figure 5.7, lane d).

In the microsome preparation, two other bands of phosphorylation were also present after incubation with calcium (and were present in most experiments where incubation was in the presence of lanthanum, see below). These two bands had an average M_r of 44,000 and 28,000 (see Figure 5.1, lane b and Figure 5.2, lane b as examples, these bands

being present in most other autoradiographs of microsomes). On Weber and Osborne gels of purified CSCPA (Figure 5.8, lane a) a band of M_r 140,000 was evident, together with bands at M_r 95,000 77-70,000, 53,000, 44,000, 30,000, and 25,000. As suggested elsewhere (Chapters 4 and 6) the lower M_r bands may be proteolysis products of the intact CSCPA. It is possible that the phosphorylated bands at 44,000 and 30,000 M_r on the autoradiographs of microsome preparations may be proteolysis products of the intact CSCPA. Further evidence enabling tentative identification of these may be gained from the effects of isotopic dilution of [γ^{32}P]-ATP and hydroxylamine treatment. After isotopic dilution of [γ^{32}P]-ATP phosphorylation at M_r 44,000 remained in three (Figure 5.2, lane g; Figure 5.3, lane h; Figure 5.6, lane h) out of four experiments (Figure 5.1, lane g being the exception). In all experiments, this phosphorylated band remained after treatment with hydroxylamine (Figure 5.1, lane h; Figure 5.2, lane h; Figure 5.3, lane f; Figure 5.6, lane e). Therefore, the phosphorylation was not turning over rapidly, nor was an acyl-phosphate intermediate formed. These two results would suggest that phosphorylation at M_r 44,000 in microsomes did not arise from a proteolysis product of the intact CSCPA. An alternative possibility was that it was the result of protein kinase activity. The phosphorylated band at about 30,000 M_r was absent after isotopic dilution of [γ^{32}P]-ATP in every experiment (Figure 5.1, lane g; Figure 5.2, lane g; Figure 5.3, lane h; Figure 5.4, lane h). Treatment with hydroxylamine did not abolish it (Figure 5.1, lane h; Figure 5.2, lane h; Figure 5.3, lane f; Figure 5.6, lane e). These results suggest that it was turning over rapidly, but an acyl-phosphate bond was probably not formed. This phosphorylation probably did not represent a proteolytic breakdown product of the intact CSCPA. Given that the proteins contained in the microsome preparation are likely to be diverse, it is not surprising that other bands of phosphorylation were present in the autoradiographs. Bands of phosphorylation at 44,000 and 30,000 M_r were not diminished in the presence of EGTA (Figure 5.1, lanes e and f; Figure 5.2, lanes e and f; Figure 5.3, lanes c-e; Figure 5.6, lanes b-d; Figure 5.9, lanes b, e, g, i; Figure 5.10, lanes

b, e, g, i) Phosphorylation was therefore probably not calcium-dependent (see section 5:2:3 for further results concerning EGTA effects).

Also apparent on the autoradiographs was a band of phosphorylation at or below the dye front. This was probably due to unincorporated [γ^{32}P]-ATP or released ^{32}P.

5.2.2 Effects of calmodulin and lanthanum

There was no reproducible effect of addition of calmodulin (0.05 μM) on phosphorylation at 140,000 M_r in microsome preparations (compare lanes a and b in Figure 5.1; Figure 5.2, lanes a and b; Figure 5.3, lanes a and b). However, in the presence of lanthanum, increased band intensity was observed as compared to incubation in the presence of calcium (Figure 5.1, lanes d and b; Figure 5.2, lanes d and b). Addition of calmodulin to samples incubated with lanthanum resulted in further enhancement of band intensity at 140,000 M_r (Figure 5.1, lanes c and d; Figure 5.2, lanes c and d). The same results were found for purified CSCPA. In the presence of calcium and calmodulin, there was no consistent enhancement of phosphorylation at 140,000 M_r as compared with phosphorylation in the presence of calcium only (Figure 5.4, lanes a and b, apparent increased intensity in lane a being a result of accidental overloading and not found in the replicate experiments; Figure 5.5, lanes a and b; Figure 5.7, lanes a and b). With addition of lanthanum, slightly increased band intensity was observed (clearest in Figure 5.4, lane d compared with lane b; also Figure 5.5, lanes d and b). Inclusion of calmodulin in samples with lanthanum resulted in further increase of band intensity (seen most clearly in Figure 5.4, lanes c and d; Figure 5.5, lanes c and d).

5.2.3 Effects of EGTA

Incubation of samples of purified CSCPA with 500 μM EGTA resulted in the absence of phosphorylation at 140,000 M_r, whether in the presence or absence of calmodulin (Figure 5.4, lanes e and f; Figure 5.5, lanes e and f; Figure 5.7, lane c). The concentration

of calcium in the assay with EGTA was less than $1x10^{-7}$ M (as determined by calcium sensitive electrode, this value being at the limit of the sensitivity range of the electrode).

Results obtained for the effect of EGTA on microsome preparations, however, were unexpected. Microsome samples (solubilised with 0.1 mg Triton X-100/mg protein, as was routine in these experiments) were incubated in the presence of 500 μM EGTA. In every experiment carried out, there was a band of phosphorylation at 153,000 M_r, but none at 140,000 M_r in the presence or absence of added calmodulin (Figure 5.1, lanes e and f; Figure 5.2, lanes e and f; Figure 5.3, lane e; Figure 5.6, lane d; Figure 5.9, lane b; Figure 5.10, lane b). The free calcium concentration in the microsome assays with 500 μM EGTA was less than $1x10^{-7}$ M (as determined by calcium sensitive electrode). Attempts were made to incubate microsomes with varying amounts of added calcium and EGTA. However, measurements of free calcium were indicative of the fact that EGTA is a very effective chelator of calcium, since in each of the calcium/EGTA combinations used, the free calcium was measured at less than $1x10^{-7}$ M. In each case, phosphorylation still persisted at 153,000 M_r (50 μM calcium and 500 μM EGTA, Figure 5.3, lane c and Figure 5.6, lane b; 500 μM calcium and 500 μM EGTA, Figure 5.3, lane d and Figure 5.6, lane c).

The 153,000 M_r phosphorylated band present when EGTA was included in the reaction medium was abolished after isotopic dilution of the [γ^{32}P]-ATP (Figure 5.3, lane g; Figure 5.6, lane g) and was diminished after treatment with hydroxylamine (Figure 5.9, lane c; Figure 5.10, lane c). The effect of calmodulin on the intensity of this band was not consistent; in Figure 5.1, lanes e and f the phosphorylated band appeared more intense in the presence of calmodulin, but the opposite appeared to occur in Figure 5.2, lanes e and f.

A further possibility for persistence of phosphorylation was that EGTA did not chelate out all the calcium in the immediate environment of the membrane-bound

CSCPA. To examine whether this possibility may have arisen from insufficient permeabilisation of the microsomes, a series of permeabilisation and solubilisation experiments were carried out. Microsomes solubilised with 0.1 mg Triton X-100/mg protein were disrupted by means of sonication with a tip sonicator for 3 minutes on ice in 15 second bursts, vigorous vortex-mixing for 10 minutes at 4°C or freezing at -20°C followed by rapid thawing to 4°C. After these three types of permeabilisation, there was a band of phosphorylation at 140,000 M_r with calcium, and at 153,000 M_r with EGTA (Figure 5.9, lanes a and b, d-i). The results obtained from freeze/thawing maize microsomes two, three or four times are depicted in Figure 5.10 (lanes a, b, d-i). The freeze/thaw cycles did not alter the pattern of phosphorylation, there being phosphorylation at 140,000 M_r in the presence of calcium and at 153,000 M_r with EGTA. This autoradiograph (Figure 5.10) also showed two other phosphorylated bands. An apparent doublet of bands (M_r 140,000 and about 153,000) was visible in the samples incubated with calcium. These were present in the control (not freeze/thawed) lane (Figure 5.10, lane a) as well as in freeze/thawed samples (Figure 5.10, lanes d, f and h). Reasons for appearance of this doublet were not entirely clear, especially since it was not a consequence of freeze/thaw treatment. It was also not clear why the band of M_r approximately 100,000 was present on this autoradiograph for the samples incubated with EGTA, whether or not freeze/thaw treatment had been carried out (Figure 5.10, lanes b, e, g and i; also Figure 5.9, lane i).

A further approach to the possibility that EGTA was not chelating calcium in the immediate environment of the CSCPA in microsomes was to investigate the effects of alternative detergents to solubilise the CSCPA. This was unsuccessful as solubilisation of microsomes with 1 mg Triton X-100/mg protein still yielded phosphorylation at 140,000 M_r with calcium and at 153,000 M_r with EGTA (Figure 5.11, lanes c and d), whilst use of 1 mg sodium deoxycholate/mg protein resulted in absence of phosphorylation, both in the presence of calcium and of EGTA (Figure 5.11, lanes e and f).

To investigate whether the maize CSCPA underwent a mobility shift on SDS-PAGE in the presence of EGTA (which could explain the appearance of the 153,000 M_r band), samples of purified CSCPA were electrophoresed on pH 6.0 Weber and Osborne gels in the presence and absence of EGTA, and the gels stained for protein. The EGTA eluted peak from the calmodulin-affinity column was divided into two, one half was brought to 10 mM $CaCl_2$ as normal and the other left in EGTA as eluted. As depicted in Figure 5.8, there was no apparent difference in M_r of any of the bands in samples incubated with EGTA (lane b) or with calcium (lane a).

Figure 5.1: Formation of phosphorylated intermediates in microsomes.

Microsomes phosphorylated in the presence of 1 μCi [γ^{32} P]-ATP: autoradiograph of 5% pH 6.0 SDS polyacrylamide gel.

Lane a: microsomes phosphorylated for 15 seconds on ice in the presence of 50 μM added calcium plus 0.05 μM calmodulin.

Lane b: as lane a, without calmodulin.

Lane c: as lane a, but with 50 μM $LaCl_3$ in place of calcium.

Lane d: as lane c without calmodulin.

Lane e: as lane a, but with 500 μM EGTA in place of calcium.

Lane f: as lane e, but without calmodulin.

Lane g: as lane a, but with 5 mM unlabelled ATP added for 1 minute after phosphorylation.

Lane h: as lane a, but pelleted membrane preparation washed with 50 mM hydroxylamine in 100 mM MES/KOH pH 6.0 prior to electrophoresis.

Note concerning relative molecular mass markers:
For each separation of polypeptides which had associated radioactivity, one lane of the gel contained M_r markers. These were excised from the gel before autoradiography, and stained with Coomassie Blue dye. It was found that these M_r markers often ran anomalously on the acidic gels. As a consequence, the experiments were repeated several times, and the values shown for the M_r of the bands of phosphorylation are the mean values obtained. Consequently, in the following figures (Figures 5.1-5.7, 5.9-5.11) there is not always perfect correlation between the positions of the M_r markers indicated on the left hand side and the values indicated on the right hand side for the M_r of the bands of phosphorylation.

this and
In all the following figures (5.2-5.11), samples for electrophoresis were incubated in SDS sample buffer containing PMSF and Chymostatin without incubation at 100°C.

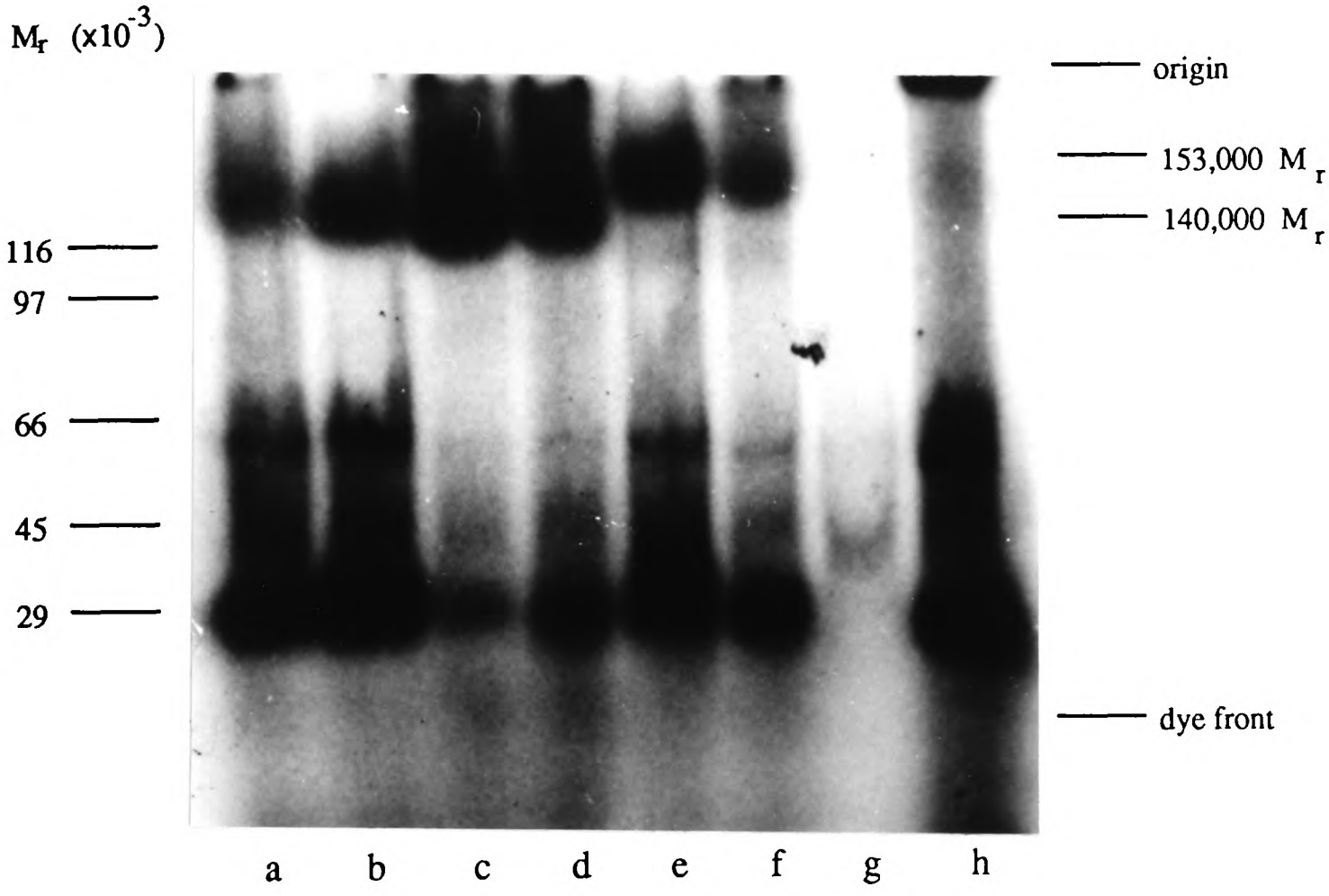

M_r (x10^{-3})
116
97
66
45
29
origin
153,000 M_r
140,000 M_r
dye front
a
b
c
d
e
f
g
h

Figure 5.2: Formation of phosphorylated intermediates in microsomes.

Microsomes phosphorylated in the presence of 1 μCi [γ^{32} P]-ATP: autoradiograph of 5% pH 6.0 SDS polyacrylamide gel.

Lane a: microsomes phosphorylated for 15 seconds on ice in the presence of 50 μM added calcium plus 0.05 μM calmodulin.

Lane b: as lane a, without calmodulin.

Lane c: as lane a, but with 50 μM $LaCl_3$ in place of calcium.

Lane d: as lane c without calmodulin.

Lane e: as lane a, but with 500 μM EGTA in place of calcium.

Lane f: as lane e, but without calmodulin.

Lane g: as lane a, but with 5 mM unlabelled ATP added for 1 minute after phosphorylation.

Lane h: as lane a, but pelleted membrane preparation washed with 50 mM hydroxylamine in 100 mM MES/KOH pH 6.0 prior to electrophoresis.

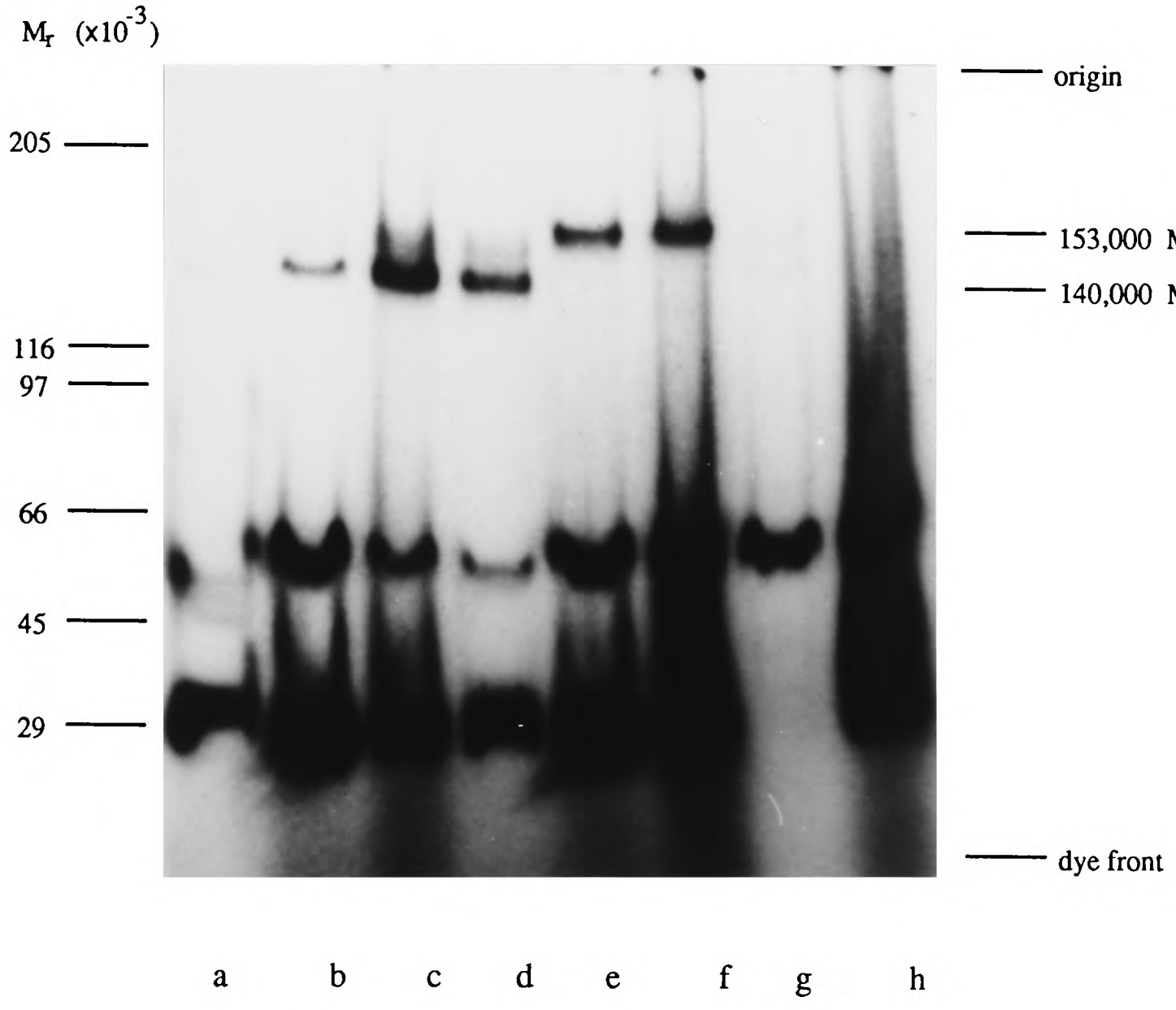

M_r (x10^{-3})
205
116
97
66
45
29
origin
153,000 M_r
140,000 M_r
dye front
a
b
c
d
e
f
g
h

Figure 5.3: Formation of phosphorylated intermediates in microsome preparations.

Phosphorylation with 5 μCi [γ^{32}P]-ATP and 2 μM ATP per assay; 5% pH 6.0 SDS polyacrylamide gel.

Lane a: microsomes phosphorylated for 15 seconds on ice in the presence of 50 μM added calcium plus 0.05 μM calmodulin.

Lane b: as lane a, without calmodulin.

Lane c: as lane b, with addition of 500 μM EGTA.

Lane d: as lane c, but 500 μM calcium

Lane e: as lane c, but with 500 μM EGTA only and no added calcium

Lane f: as lane b, but pelleted membrane preparation washed with 50 mM hydroxylamine in 100 mM MES/KOH pH 6.0 prior to electrophoresis.

Lane g: as lane a, but with 5 mM unlabelled ATP added for 1 minute after phosphorylation.

Lane h: as lane c, but with 5 mM unlabelled ATP added for 1 minute after phosphorylation.

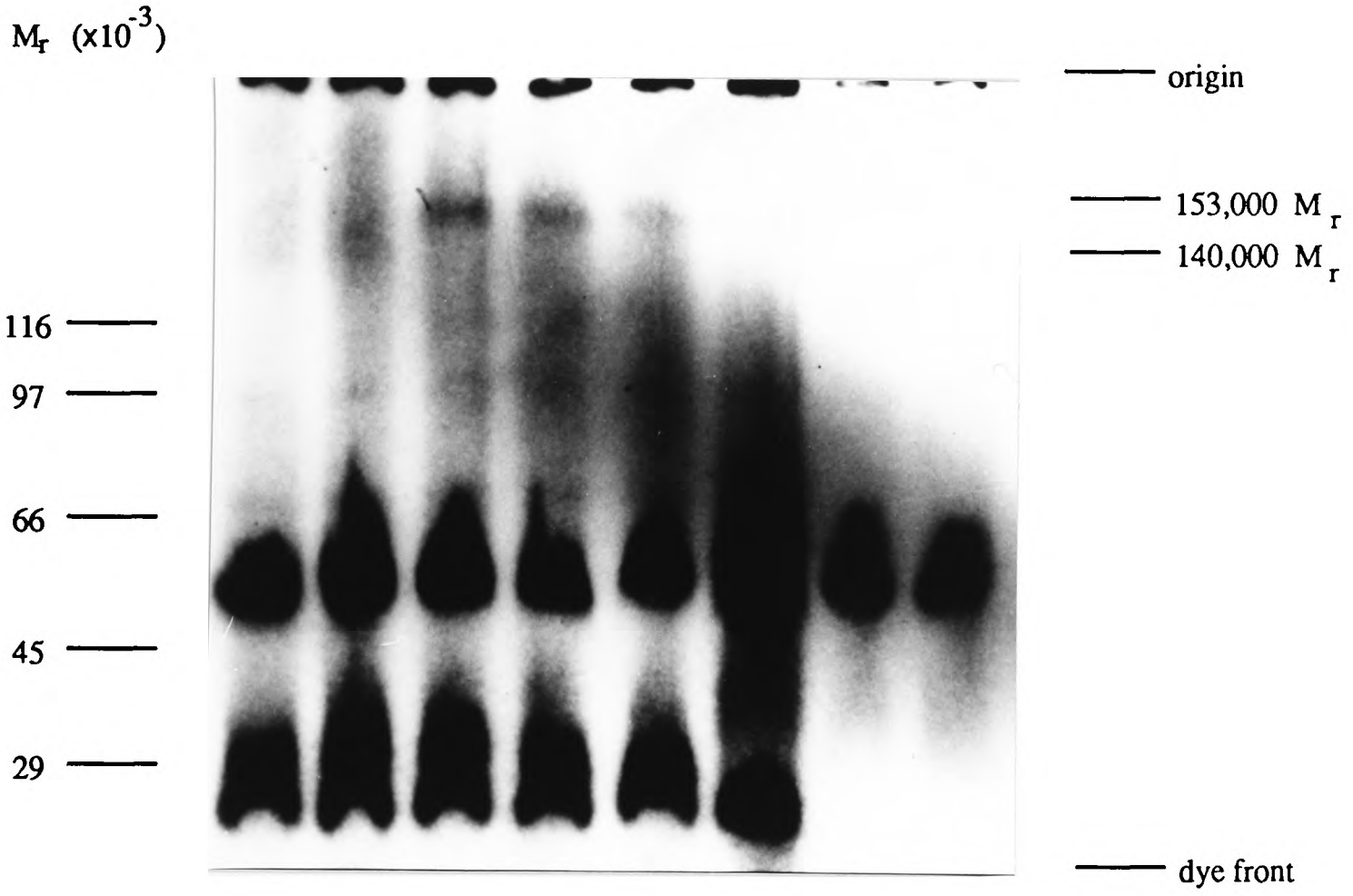

M_r (x10^{-3})
116
97
66
45
29
origin
153,000 M_r
140,000 M_r
dye front
a
b
c
d
e
f
g
h

Figure 5.4: Formation of phosphorylated intermediates by purified CSCPA.

Phosphorylation in the presence of 1 μCi [γ^{32} P]-ATP: autoradiograph of 7. 5% pH 6.0 SDS polyacrylamide gel.

Lane a: purified CSCPA phosphorylated for 15 seconds on ice in the presence of 50 μM added calcium plus 0.05 μM calmodulin.

Lane b: as lane a, without calmodulin.

Lane c: as lane a, but with 50 μM $LaCl_3$ in place of calcium.

Lane d: as lane c without calmodulin.

Lane e: as lane a, but with 500 μM EGTA in place of calcium.

Lane f: as lane e, but without calmodulin.

Lane g: as lane a, but with 5 mM unlabelled ATP added for 1 minute after phosphorylation.

Lane h: as lane a, but pelleted membrane preparation washed with 50 mM hydroxylamine in 100 mM MES/KOH pH 6.0 prior to electrophoresis.

Purification of CSCPA carried out by calmodulin-affinity chromatography. All buffers contained PMSF, and asolectin was present in the affinity column buffers. The fraction assayed was the pooled peak (see Figure 4.1).

This purification was also used in Figure 5.5.

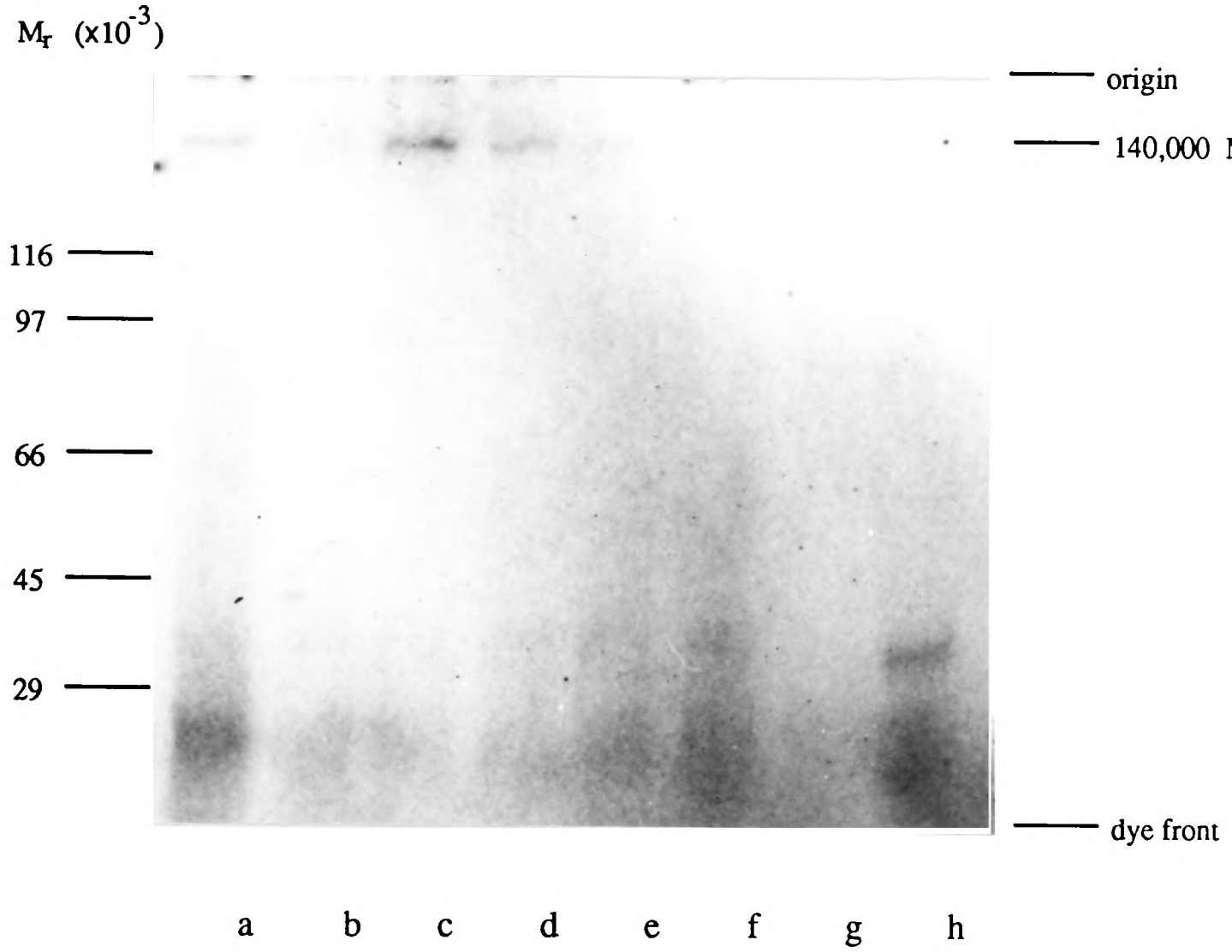

M_r (x10^-3)
origin
140,000 M_r
116
97
66
45
29
dye front
a
b
c
d
e
f
g
h

Figure 5.5: Formation of phosphorylated intermediates by purified CSCPA.

Phosphorylation in the presence of 1 μCi [γ^{32} P]-ATP: autoradiograph of 5% pH 6.0 SDS polyacrylamide gel.

Lane a: purified CSCPA phosphorylated for 15 seconds on ice in the presence of 50 μM added calcium plus 0.05 μM calmodulin.

Lane b: as lane a, without calmodulin.

Lane c: as lane a, but with 50 μM $LaCl_3$ in place of calcium.

Lane d: as lane c without calmodulin.

Lane e: as lane a, but with 500 μM EGTA in place of calcium.

Lane f: as lane e, but without calmodulin.

Lane g: as lane a, but with 5 mM unlabelled ATP added for 1 minute after phosphorylation.

Lane h: as lane a, but pelleted membrane preparation washed with 50 mM hydroxylamine in 100 mM MES/KOH pH 6.0 prior to electrophoresis.

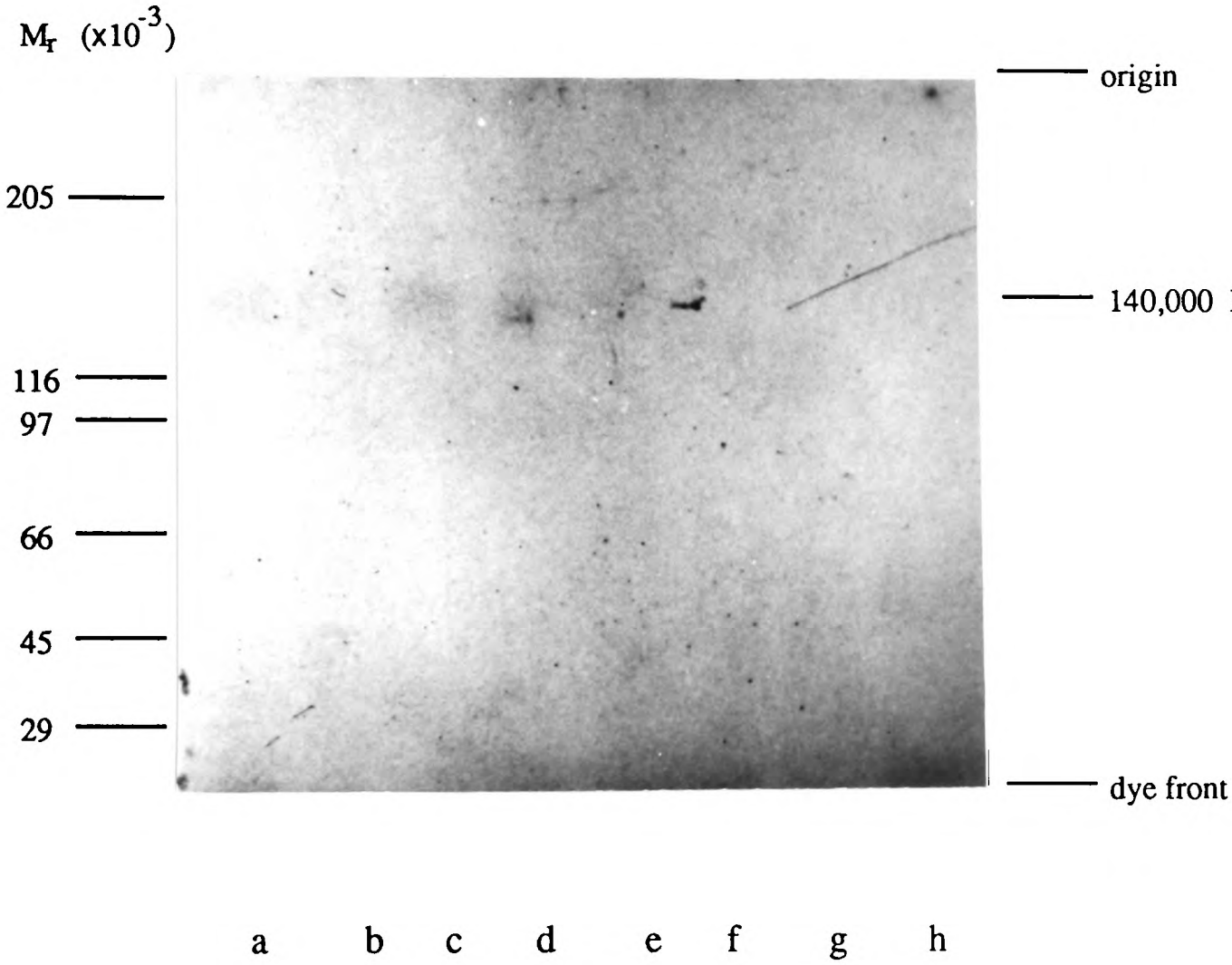

M_r ($x10^{-3}$)
205
116
97
66
45
29
origin
140,000 M_r
dye front
a
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c
d
e
f
g
h

Figure 5.6: Formation of phosphorylated intermediates in microsomes.

Incubation with 5 μCi [γ_{32} P]-ATP and 2 μM ATP; autoradiograph of 5% pH 6.0 SDS polyacrylamide gel.

Lane a: microsomes phosphorylated for 15 seconds on ice in the presence of 50 μM added calcium.

Lane b: as lane a, but with 500 μM EGTA added.

Lane c: as lane a, but with 500 μM $CaCl_2$ and 500 μM EGTA.

Lane d: as lane a, but without calcium and with 500 μM EGTA.

Lane e: as lane a, but pelleted membrane preparation washed with 50 mM hydroxylamine in 100 mM MES/KOH pH 6.0 prior to electrophoresis.

Lane f: as lane e, but pelleted membranes washed with 100 mM MES/KOH only.

Lane g: as lane d, but with 5 mM unlabelled ATP added for 1 minute after phosphorylation.

Lane h: as lane a, but 5 mM unlabelled ATP added for 1 minute after phosphorylation.

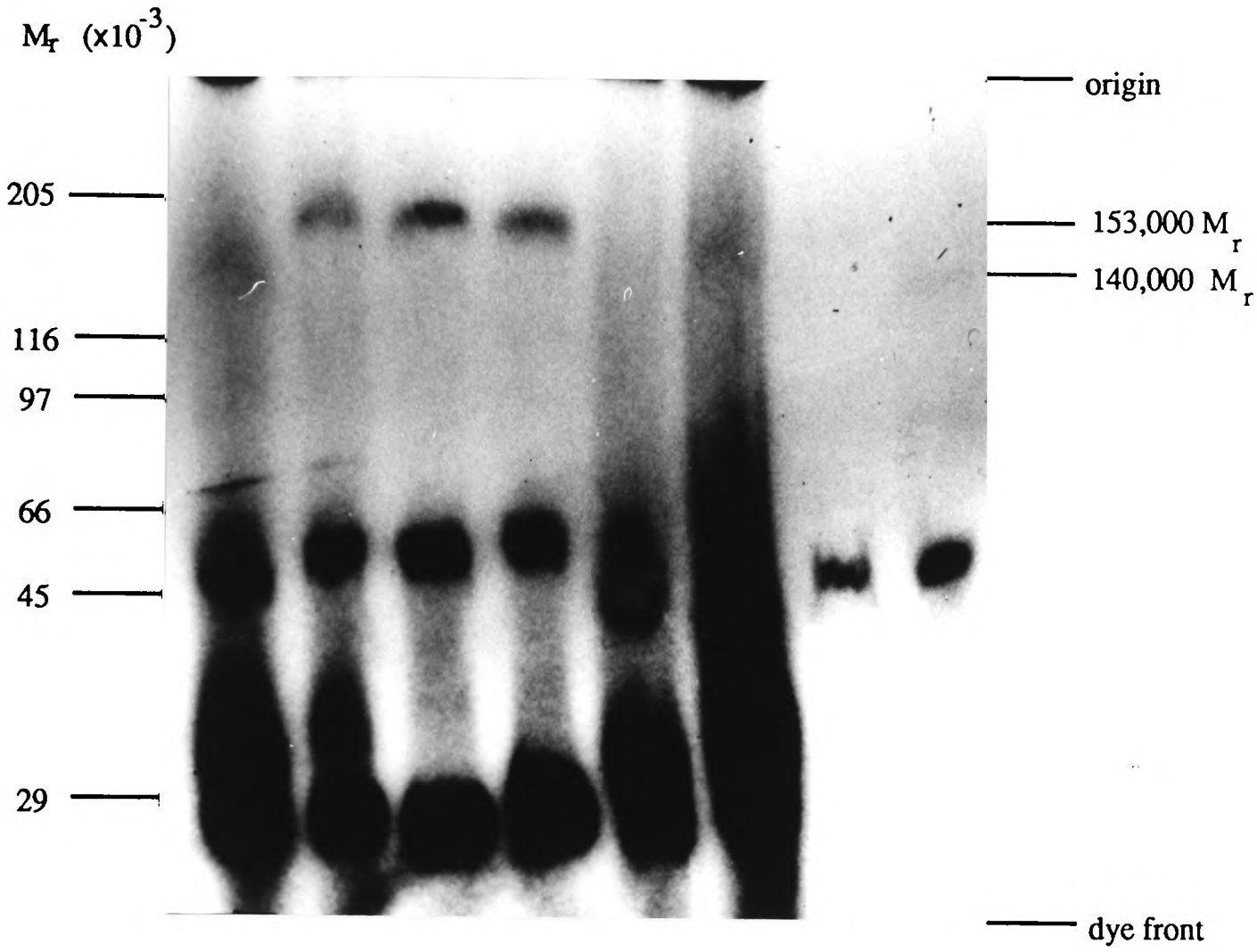

M_r ($x10^{-3}$)
205
116
97
66
45
29
origin
153,000 M_r
140,000 M_r
dye front
a
b
c
d
e
f
g
h

Figure 5.7: Formation of phosphorylated intermediates by purified CSCPA.

Phosphorylation in the presence of 5 μCi [γ^{32} P]-ATP and 2 μM ATP; autoradiograph of 5% pH 6.0 SDS polyacrylamide gel.

Lane a: purified CSCPA phosphorylated for 15 seconds on ice in the presence of 50 μM added calcium plus 0.05 μM calmodulin.

Lane b: as lane a, without calmodulin.

Lane c: as lane a, but with 500 μM EGTA in place of calcium.

Lane d: as lane a, but pelleted protein washed with 50 mM hydroxylamine in 100 mM MES/KOH prior to electrophoresis.

Lane e: as lane a, but with 5 mM unlabelled ATP added for 1 minute after phosphorylation.

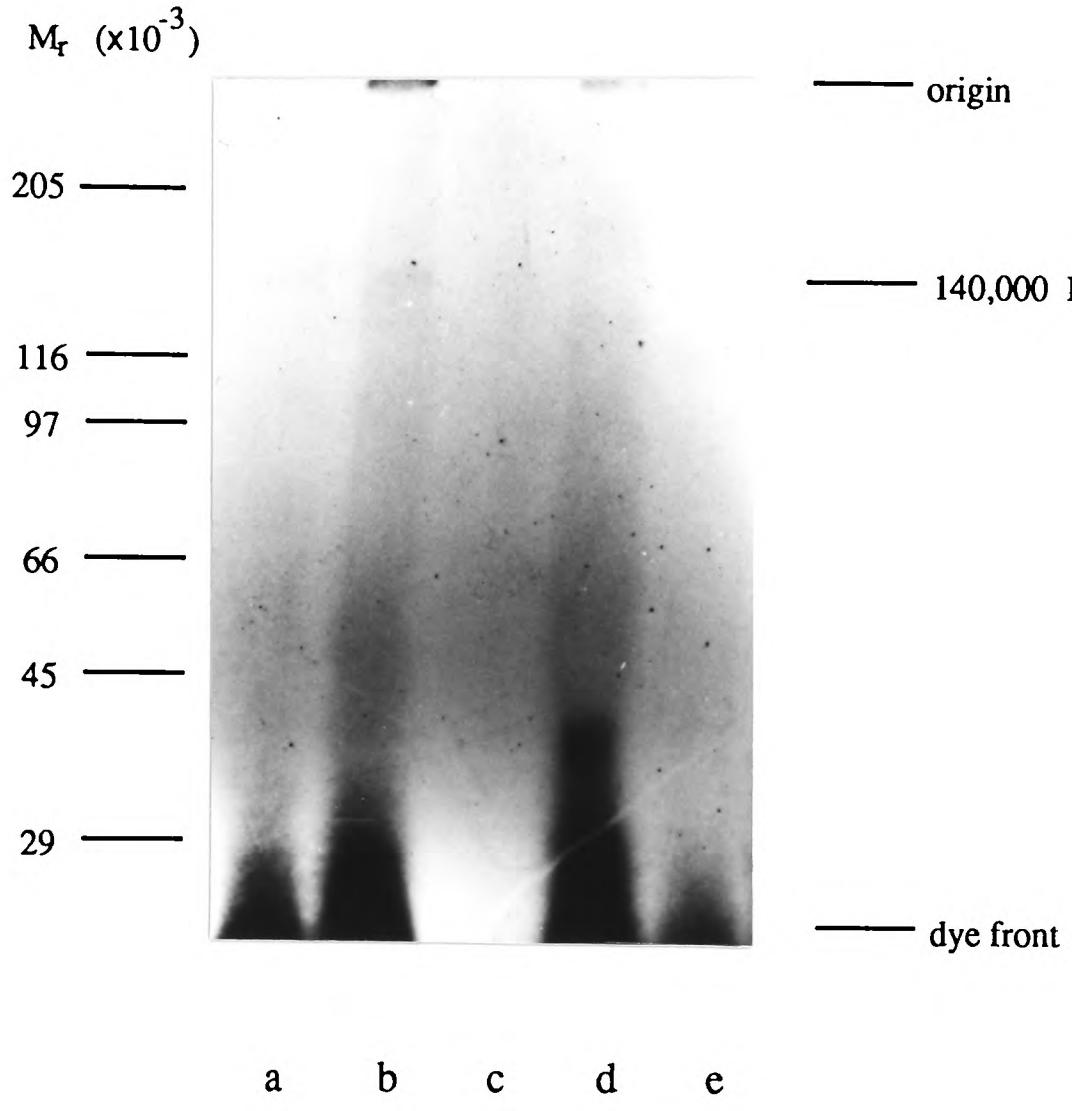

M_r ($x10^{-3}$)
205
116
97
66
45
29
origin
140,000 M_r
dye front
a
b
c
d
e

Figure 5.8: Effect of EGTA on purified CSCPA.

pH 6.0 SDS-polyacrylamide gel stained for protein with Coomassie Blue dye followed by silver enhancement.

Lane a: CSCPA in the presence of 10 mM $CaCl_2$.

Lane b: CSCPA in the presence of EGTA as eluted from the calmodulin-affinity column.

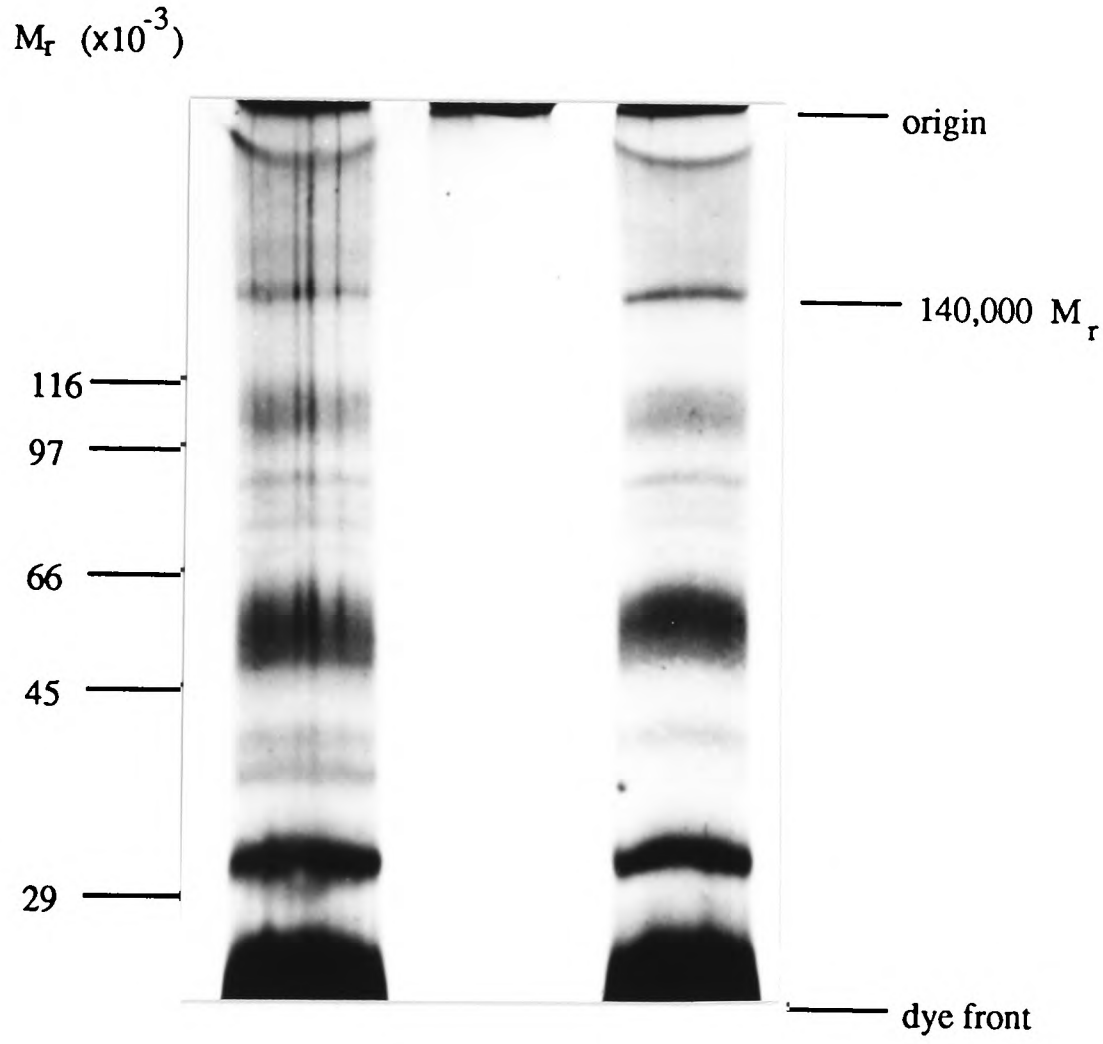

M_r (x10^{-3})
116
97
66
45
29
origin
140,000 M_r
dye front
a
b

Figure 5.9: Effect of permeabilising treatments on formation of phosphorylated intermediates in microsomes.

Phosphorylation with 1 μCi [γ ^{32}P]-ATP per assay: autoradiograph of 5% SDS-polyacrylamide gel.

Lane a: microsomes phosphorylated with 50 μM added calcium.

Lane b: as lane a, but with 500 μM EGTA in place of calcium.

Lane c: as lane b, but pelleted membranes washed with 50 μM hydroxylamine in 100 mM MES/KOH pH 6.0 prior to electrophoresis.

Lane d: microsomes phosphorylated with 50 μM calcium after sonication with 0.1 mg Triton X-100/mg protein.

Lane e: as lane d, but 500 μM EGTA in place of calcium.

Lane f: microsomes phosphorylated as lane a, but vortexed for 10 minutes.

Lane g: as lane f, but 500 μM EGTA in place of calcium.

Lane h: microsomes phosphorylated as lane a, but after two freeze/thaw cycles.

Lane i: as lane h, but with 500 μM EGTA in place of calcium.

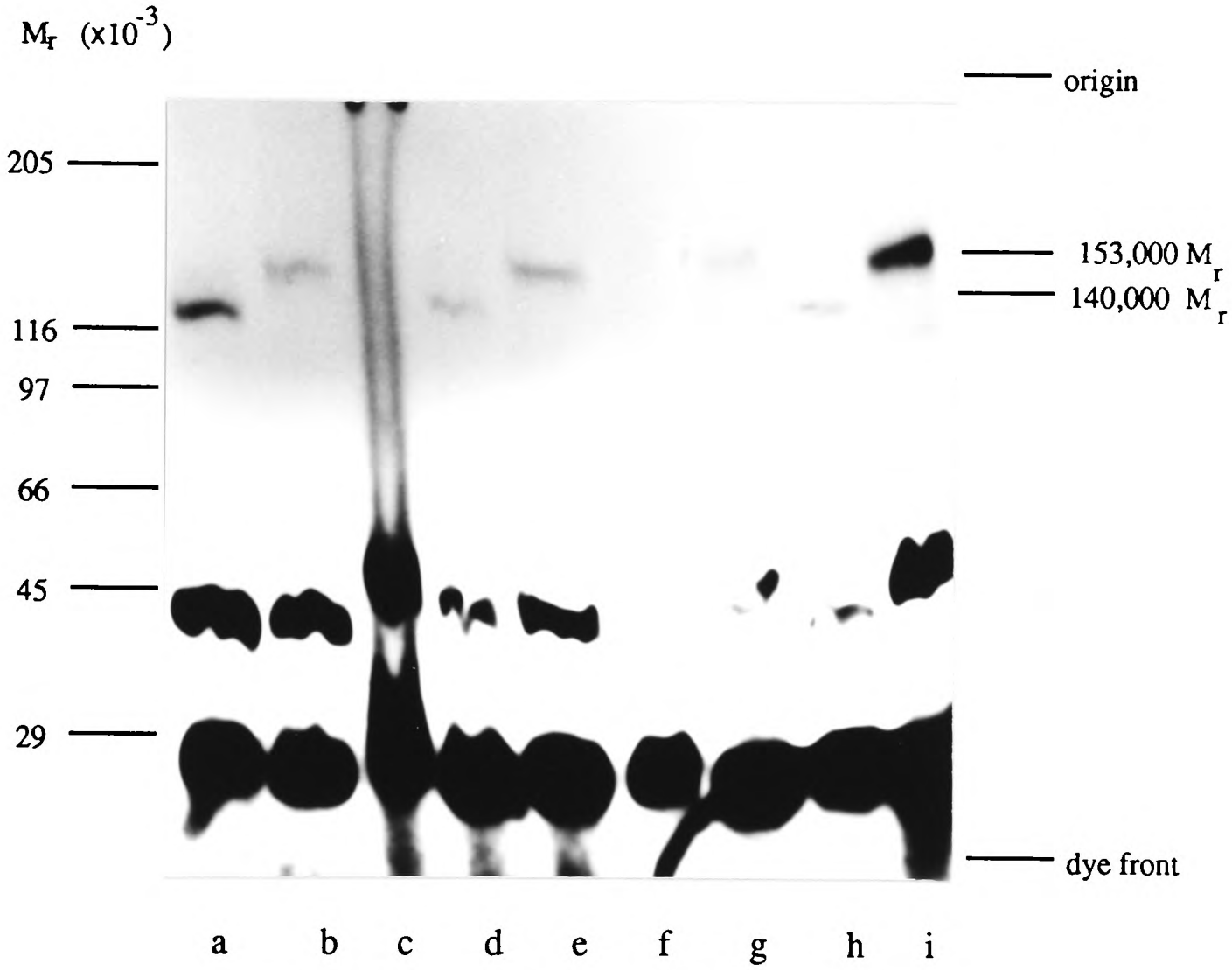

M_r ($x10^{-3}$)
205
116
97
66
45
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origin
153,000 M_r
140,000 M_r
dye front
a
b
c
d
e
f
g
h
i

Figure 5.10: Effect of freeze/thaw treatments on formation of phosphorylated intermediates in microsomes.

Phosphorylation with 1 μCi [γ ^{32}P]-ATP per assay: autoradiograph of 5% SDS-polyacrylamide gel.

Lane a: microsomes phosphorylated with 50 μM added calcium.

Lane b: as lane a, but with 500 μM EGTA in place of calcium.

Lane c: as lane b, but pelleted proteins washed with 50 μM hydroxylamine in 100 mM MES/KOH pH 6.0 prior to electrophoresis.

Lane d: as lane a but microsomes phosphorylated after two freeze/thaw cycles.

Lane e: as lane d, but 500 μM EGTA in place of calcium.

Lane f: as lane d, but phosphorylation after three freeze/thaw cycles.

Lane g: as lane f, but 500 μM EGTA in place of calcium.

Lane h: as lane d, but phosphorylation after four freeze/thaw cycles.

Lane i: as lane h, but with 500 μM EGTA in place of calcium.

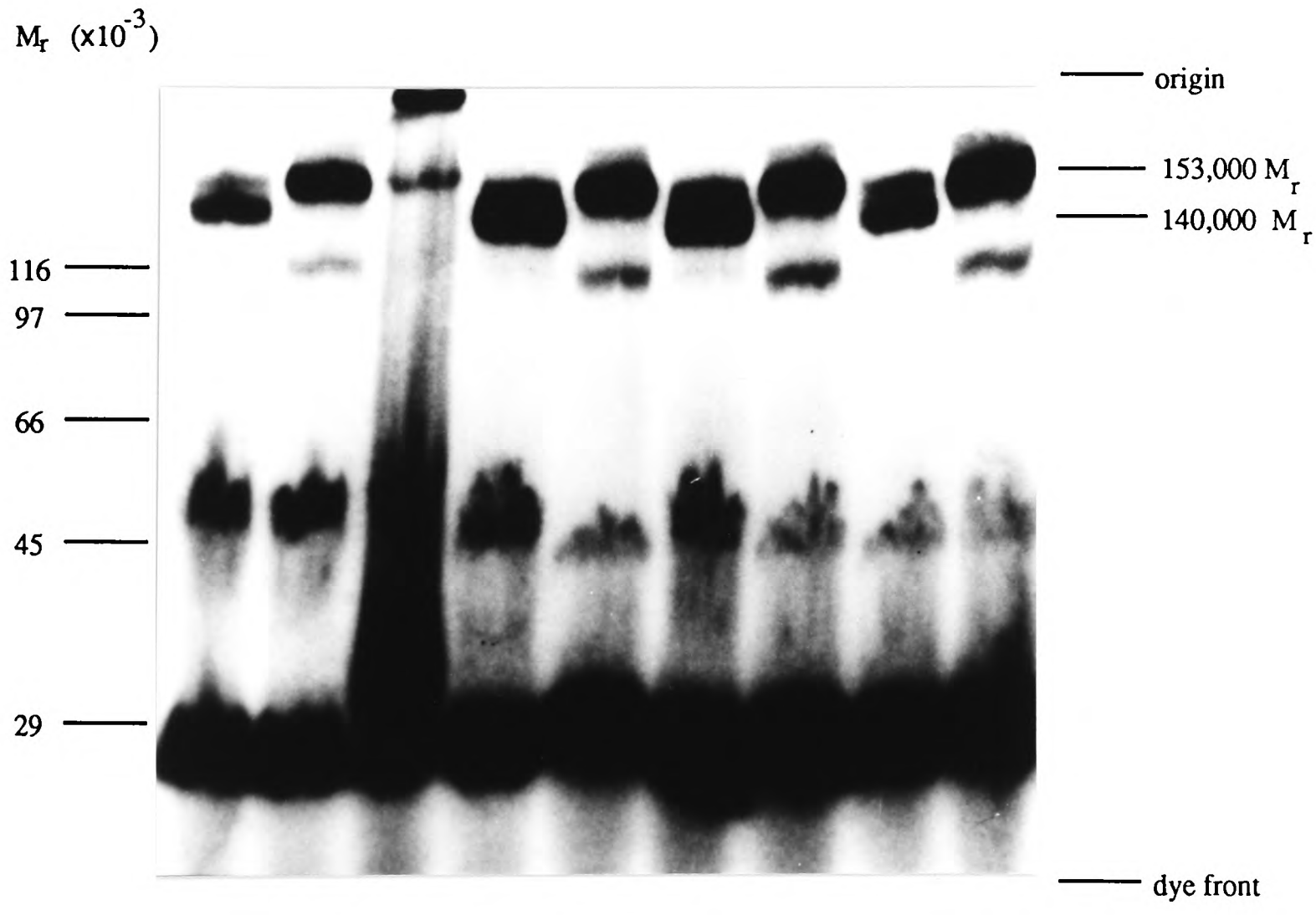

M_r (x10^{-3})
116
97
66
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29
origin
153,000 M_r
140,000 M_r
dye front
a
b
c
d
e
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i

Figure 5.11: Effect of solubilisation procedures on formation of phosphorylated intermediates in microsomes.

Phosphorylation with 1 μCi [γ ^{32}P]-ATP per assay: autoradiograph of 5% SDS-polyacrylamide gel.

Lane a: microsomes solubilised with 0.1 mg Triton X-100/mg protein and phosphorylated in the presence of 50 μM $CaCl_2$.

Lane b: as lane a, but with 500 μM EGTA in place of calcium.

Lane c: as lane a, but microsomes solubilised with 1.0 mg Triton X-100/mg protein.

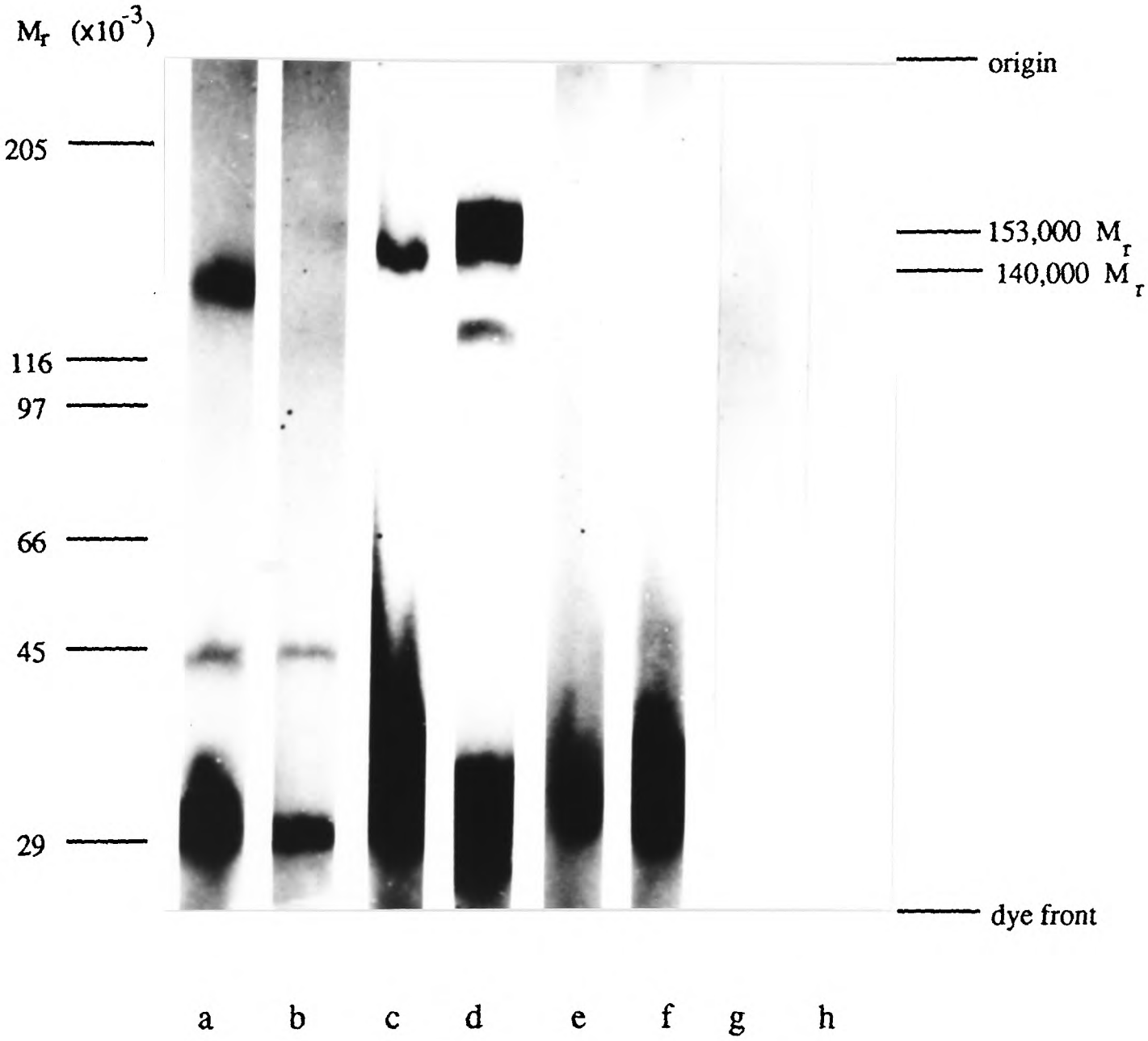
Lane d: as lane c, but 500 μM EGTA in place of calcium.

Lane e: as lane a, but microsomes phosphorylated after solubilisation with 1 mg sodium deoxycholate/mg protein and freeze/thawed once.

Lane f: as lane e, but 500 μM EGTA in place of calcium.

Lane g: as lane a, but microsomes boiled for 2 minutes prior to phosphorylation.

Lane h: as lane g, but with 500 μM EGTA in place of calcium.


M_r ($x10^{-3}$)
205
116
97
66
45
29
origin
153,000 M_r
140,000 M_r
dye front
a
b
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d
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h

5.3 DISCUSSION

The results presented in the preceding section of this chapter demonstrated that a polypeptide of 140,000 M_r in a microsomal fraction and a clamodulin-affinity purified extract from maize displayed the properties expected of a P-type ion translocating ATPase. These results also provided further confirmation that the 140,000 M_r polypeptide described in Chapter 4 was the equivalent in maize of the CSCPA found in erythrocytes.

Purified maize CSCPA formed a phosphorylated band at 140,000 M_r on acidic gels when incubated with [γ^{32}P]-ATP for 15 seconds on ice (Figures 5.4, 5.5). This band was also present when microsomal preparations were treated similarly (Figures 5.1, 5.2, 5.6, 5.9, 5.10, 5.11). A chase with unlabelled ATP and treatment with hydroxylamine abolished this band in all cases (Figures 1-7). The phosphorylated intermediates of P-type ATPases turn over rapidly and are sensitive to hydroxylamine treatment (see sections 5:1:1-3). These results therefore indicate that the 140,000 M_r band is a P-type ATPase.

From the measurements of free calcium made in the assays, it would appear that purified CSCPA underwent phosphorylation at concentrations greater than around 10^{-6} M, but at values much lower than this, phosphorylation was prevented. These results were consistent with the estimated free calcium concentrations in plant cells (see Chapter 1). At low calcium concentrations believed to be present in unstimulated cells (in the order of 10^{-7} M) the CSCPA was inactive. At calcium concentrations greater than this, the CSCPA was activated in terms of phosphorylated intermediate formation, and therefore would be functional to remove free calcium from the cytoplasm. The results presented for formation of phosphorylated intermediates in microsome preparations were also consistent with results obtained in the study of phosphorylation in erythrocyte membranes, with the exception of the EGTA effects (see below for further discussion of this).

In both purified CSCPA and microsomal fractions, it was found that addition of

lanthanum brought about an enhancement of intensity of the band at 140,000 M_r (Figures 5.1, 5.2, 5.4, 5.5). This further indicated that the 140,000 M_r band was a P-type ATPase, since it has been established that the effect of lanthanum (for references see section 5:1:2) is to block dephosphorylation of P-type ATPases, whilst permitting phosphorylation to occur.

The effect of calmodulin and lanthanum in microsome and purified CSCPA samples indicated that calmodulin increased the rate at which turnover of the phosphorylated intermediate took place. This may be deduced as follows. The effects of calmodulin and lanthanum may be considered in terms of the simple model (section 5:1) for phosphorylation from Muallem and Karlish (1980). Luterbacher and Schatzmann (1983) suggested that the site of action of lanthanum was at the $E_1 \sim P \rightleftharpoons E_2\text{-}P$ transition. The band of phosphorylation of the CSCPA on the autoradiographs presented in this chapter represents both $E_1 \sim P$ and E_2-P. In the presence of lanthanum, calmodulin acts to increase the foward rate of the reaction $E_1 + ATP \rightleftharpoons E_1 \sim P$. Since there was no increased phosphorylation observed in samples incubated with calcium and calmodulin, it may be suggested that calmodulin increased the rate of dephosphorylation as well as the rate of phosphorylation. This concept is consistent with studies on the erythrocyte membrane CSCPA, where calmodulin has been suggested to act by enhancing the rate of turnover of the dephosphorylated state (Muallem and Karlish, 1980; Rega and Garrahan, 1980; Jeffrey *et al.* , 1981; Allen *et al.* , 1987). Such an action of calmodulin in regulating the CSCPA correlates with the proposal that, when $_p[Ca]_{cyt}$ rises, calcium binds to calmodulin, which then interacts with the calcium pump to increase the rate at which calcium is removed from the cytoplasm. If calmodulin acted only to increase the rate of phosphorylation, dephosphorylation proceding at the normal rate, then the capacity and efficiency of the CSCPA in removing calcium would be impaired.

There was a surprisingly small number of phosphorylated bands in microsome preparations incubated under the conditions described here. Most noticeable was the lack of

a phosphorylated band at M_r approximately 100,000 in the majority of the preparations (Fig. 5.9, lane a and Fig. 5.10, lanes b, e, g, and i being possible exceptions to this). The presence of such a band might be expected as a result of the proton-pumping ATPase although experiments were not conducted at its pH optimum (pH 6.5, Leonard *et al.* , 1973; Hodges and Leonard, 1974). The intensity of the phosphorylated bands in most of the experiments with microsomes was also surprising. These autoradiographs were developed after only 24 hours exposure whereas autoradiographs of purified CSCPA required considerably longer. The amount of calmodulin-stimulated, calcium pumping ATPase contained in about 200 μg of total microsomal protein loaded per lane on the gels is likely to be at least five times less than that contained in one lane of an autoradiograph of purified CSCPA. The intensity of phosphorylation in the microsome samples may arise because the membrane environment of the CSCPA is of importance in producing full activity in terms of phosphorylated intermediate formation.

Purified CSCPA did not form a phosphorylated intermediate at calcium concentrations lower than about $1x10^{-7}$ M (as determined by calcium-sensitive electrode, section 5:2:3). This result is in agreement with those obtained for purified erythrocyte CSCPA (Niggli *et al.* , 1979b; Allen *et al.* , 1987), and indicates that purified maize CSCPA may have a similar reaction mechanism, in which activity of the E_1 state is expressed only in the presence of calcium (shown for red blood cells by Katz and Blostein, 1975; Rega and Garrahan, 1975). Results obtained from phosphorylated intermediate formation by microsomes were more difficult to interpret. In assays with microsomes, added EGTA reduced the calcium concentration to less than 10^{-7} M. Although the band of phosphorylation at 140,000 M_r was abolished, another appeared at 153,000 M_r which showed the properties of a P-type ATPase (section 5:2:3). Three possibilities may be suggested to explain this pattern of phosphorylation. The first is that EGTA effectively chelated out all calcium in the medium and in the immediate environment of the CSCPA, such that formation of a phosphorylated intermediate by the CSCPA was

inhibited. The band at 153,000 M_r may then be suggested to have been due to another ion-translocating ATPase which was active (in this *in vitro* situation) only at extremely low calcium concentrations (less than 1 x 10^{-7} M). The physiological relevance of such an ion transporter remains questionable, although translation of this result to an *in vivo* situation will be open to debate until more information can be gained about this phosphorylation at 153,000 M_r.

A second possibility for phosphorylation in the presence of EGTA may be that in all the experiments described above sufficient tightly bound calcium was retained in the vicinity of the CSCPA such that phosphorylation could occur. Attempts to remove tightly sequestered calcium from the microsome preparation by detergent treatments and/or freeze/thaw cycles are described in section 5:2:3. Experiments on phosphorylation in erythrocytes often involve such treatments (Katz and Blostein, 1975; Rega and Garrahan, 1975; Szasz *et al.* , 1978). However, after use of increased concentrations of Triton X-100, phosphorylation was still present at 153,000 M_r, as was the case for the freeze/thaw treatment and sonication or vortexing of the samples (Figures 5.9, 5.10, 5.11). Use of sodium deoxycholate was also unsuccessful, as in this case no phosphorylation was present at any M_r value (Figure 5.11).

If the 153,000 M_r band was the CSCPA active due to sequestered calcium, it is necessary to explain the apparent shift in M_r which occured on incubation with EGTA. It is unlikely that this difference arose from inaccuracies in the estimations, since mobilities of other polypeptides were not affected. However, given the consistency of this finding, it is suggested that the difference in M_r between samples incubated with calcium and with EGTA was a real phenomenon. Various reports exist of other calcium-stimulated proteins displaying a calcium-dependent mobility shift during electrophoresis (for example, calcium dependent protein kinases, Battey and Venis, 1988; Bögre *et al.* , 1988) although these reports were for purified, not membrane bound, protein kinase. The purified maize CSCPA did not show a calcium-dependent mobility shift (section 5:2:3,

Figure 5.8). Examination of three early reports on phosphorylated intermediate formation in erythrocyte membranes may have some bearing on this result. Knauf *et al.* (1974) produced autoradiographs of red cell ghosts phosphorylated in the presence of calcium that showed a doublet of bands of M_r 150,000 and 142,000 (these being absent when calcium was omitted from the assay, however). Katz and Blostein (1975) reported formation of a 150,000 M_r phosphorylated intermediate in erythrocyte membranes that was only partially dephosphorylated after addition of excess EGTA. Finally, Szasz *et al.* (1978) did not show a decrease of phosphorylation in the presence of EGTA. The phosphorylated intermediate formed with EGTA appeared to undergo a shift to a higher M_r value, although an M_r value was not ascribed and no comment about this result was made in the publication. A further point to note is that the isolated erythrocyte membranes in these three studies were not treated with detergent. A comment made by Sarkadi (1980) may be of relevance here, in that EDTA or EGTA chases only caused rapid dephosphorylation of the erythrocyte calcium pump phosphorylated intermediate if this was formed in the presence of calcium and magnesium. In the absence of magnesium (as Sarkadi states was the case in the experiments of Szasz *et al.*) phosphorylation does occur, but dephosphorylation after addition of EGTA or EDTA was slow. This, however, should not be the case for the experiments presented in this chapter, where addition of EGTA to the phosphorylation medium should reduce the calcium to very low levels, whilst maintaining the magnesium concentration. In subsequent related publications on phosphorylation in erythrocyte membranes, these findings were not reproduced, and the membrane-bound CSCPA appeared to show calcium dependence. As suggested by Katz and Blostein (1975) another possible reason for the persistence of phosphorylation with EGTA may be that there are unknown effects of the membrane on activity of the CSCPA. If this was so for the plant CSCPA, then interesting possibilities may be raised in terms of mechanisms of regulation, which may be at variance with the majority of results obtained for the erythrocyte calmodulin-stimulated, calcium

pumping ATPase.

The third possible explanation for persistence of phosphorylation of maize microsome membranes in the presence of EGTA is that a very rapid kinase may have been present. If so, then this was unusual in that phosphorylation occured very rapidly, and only in the absence of calcium.

At present it is not feasible to resolve which of these possibilities represents the most likely explanation for the results obtained with microsomes and EGTA. Further information regarding the role of the membrane in regulating the CSCPA is needed. An alternative approach to clarifying these results would require an antibody which recognised the intact CSCPA in microsomes. This would provide an alternative means of determining the M_r of the CSCPA in microsome samples incubated with calcium and with EGTA, so detecting whether there was a calcium-dependent mobility shift. As yet such an antibody is not available; antibody 97.1 from Zürich recognised only the purified intact CSCPA (Chapter 4, section 4:2).

The studies reported here have laid the foundation for future work which may enable further details of the reaction mechanism of the CSCPA of *Zea mays* coleoptiles to be elucidated. Such a study would be particularly interesting if combined with a study of calcium-transporting ATPases in other plant tissues. In several studies carried out on calcium transport in microsomal membrane preparations it was not possible to demonstrate calmodulin stimulation (for example, Butcher and Evans, 1987a and b, for calcium transport in pea root microsomes and purified membrane fractions). It would be interesting to extract microsomes from a variety of other plant tissues, and assay for formation of phosphorylated intermediates in the presence of calcium, lanthanum and calmodulin. Such an approach may yield further evidence as to whether all plant tissues contain a calmodulin-stimulated, calcium pumping ATPase at the plasma membrane. It seems that, whilst the majority of calcium-pumping ATPases in mammalian plasma membranes are stimulated by calmodulin, there are some which are insensitive

to calmodulin, for example that of corpus luteum (Verma and Penniston, 1981). It is possible that plant plasma membranes may contain more than one type of calcium pumping ATPase. If this is so, further questions will be raised as to the mechanisms and control of calcium efflux from plant cells (see Chapter 7).

Chapter 6

PRODUCTION AND USE OF MONOCLONAL ANTIBODIES TO PURIFIED CALMODULIN-STIMULATED CALCIUM-PUMPING ATPase OF MAIZE COLEOPTILES

6.1 INTRODUCTION

6.1.1 Production of monoclonal antibodies; general considerations

The results presented in the previous three chapters may be summarised as follows:

1. In Chapter 3 calmodulin-stimulated ATPase activity and calmodulin-stimulated calcium uptake were demonstrated in a microsomal membrane fraction from maize

coleoptiles.

2. In Chapter 4 it was shown that Triton X-100 could be used to solubilise microsomal membrane proteins, and that calmodulin-binding proteins from this fraction could be separated by calmodulin-affinity chromatography. If this was carried out in the absence of the protease inhibitor phenylmethylsulphonyl fluoride (PMSF) it was possible to show calmodulin-stimulated ATPase activity in the eluted fraction. Reconstitution into asolectin liposomes was also attempted, and calmodulin-stimulated ATPase activity demonstrated. In the presence of PMSF, a polypeptide of 140,000 M_r was present after electrophoresis, of similar M_r to that of the erythrocyte CSCPA. This 140,000 M_r polypeptide was recognised by a monospecific polyclonal antibody raised against the erythrocyte CSCPA, indicating that there is immunological homology between the two calcium transporters.

3. In Chapter 5 properties of the reaction mechanisms of the CSCPA in microsome and purified preparations were investigated and it was shown that the maize CSCPA forms a transient phosphorylated intermediate which has the properties of a P-type ion translocating ATPase.

On the basis of these results it was decided that a further means by which the properties of the CSCPA could be investigated was to raise monoclonal antibodies to CSCPA purified in the absence of PMSF. The use of monoclonal antibodies to study plant antigens is a relatively new area of research (see section 6:1:2 below). There are few reports of the production of monoclonal antibodies to higher plant membrane-bound ion translocators (see section 6:3 for further discussion and references) and hence this study was designed as a preliminary investigation of the approaches required in the production of monoclonal antibodies to the maize CSCPA. The choice was made to investigate the production of monoclonal antibodies (rather than polyclonal antibodies) because of the high specificity of binding of monoclonal antibodies, and their usefulness in investigating structure/function relationships.

Since the original pioneering work of Kohler and Milstein (1975) many reviews have been published concerning the theory and techniques of monoclonal antibody production, for example, de St. Groth and Scheidegger (1980), Galfre and Milstein (1981), Goding (1983), Harlow and Lane (1988). One of the most important stages in the production of monoclonal antibodies is in the choice of initial screening method for the hybridoma supernatants containing antibodies. The choice of primary screening method depends on the nature of the antigen and the use to which the antibodies produced are to be put. In this study, the method chosen for initial screening was that of antibody capture on ELISA plates coated with antigen. The advantage of this method was that large numbers of samples could be screened with relative ease within a fairly short space of time, and periodate treatment of antigen-coated wells allowed for some characterisation of the epitope. Hybridoma supernatants which gave positive results in this assay were suitable for use in subsequent experiments to investigate the structure of the CSCPA and were also used in assays to investigate the effects of the antibodies on the ATPase activity of the CSCPA.

6.1.2 Use of monoclonal antibodies in the study of plant cells and their components

The first report in which monoclonal antibodies were raised to a plant protein was that of Leidgens *et al.* (1980) in which antibodies to 5-aminolevulinate dehydratases were produced. It was shown that these antibodies provided a useful tool to detect 'conserved domains' between 5-aminolevulinate dehydratases in various plant species (Schneider and Leidgens, 1981). The use of monoclonal antibodies to study components of plant cells is therefore a relatively new area of study, and one in which details of the methodology required are still being developed. Some examples of the uses of monoclonal antibodies in the study of plant cells are given below; this account is not intended as a complete review of the subject, but to give an indication of their use in

the study of plant membranes and enzymes.

One of the major uses of monoclonal antibodies in the study of plant cells has been in the generation of antibodies to membrane-specific, tissue-specific or determination-specific antigens. Bolwell and Northcote (1984) raised monoclonal antibodies to purified endoplasmic reticulum and Golgi-membranes. The antibodies produced recognised, by immunoblotting, several polypeptides and were shown specifically to inhibit the enzyme arabinosyl transferase (which is involved in the synthesis of the primary cell wall component arabinan). Monoclonal antibodies were raised by Norman *et al.* (1986) to a crude membrane preparation from *Nicotiana glutinosa* L. and were screened for specific detection of plasma membrane antigens by immunofluorescence. The antigens which were recognised by these antibodies were found to be of wide distribution in plasma membranes from different organs and different species. It was suggested that they could be of use as molecular markers for plasma membrane. This study was followed by a report (Fitter *et al.* , 1987) in which monoclonal antibodies raised to plasma membrane as described above were used to identify heterokaryons in protoplast fusion techniques. It was found that labelling with the antibody did not prevent callus formation and regeneration of the plantlet, and provided a means by which heterokaryons could be distinguished from unfused aggregates and homokaryons. Additional monoclonal antibodies which recognised plasma membrane antigens were raised by Hahn *et al.* (1987) using methods similar to those of Norman *et al.* (1986), although in this study protoplasts from *Nicotiana tabacum* L. were used as the immunogen. The antibodies were screened by immunofluorescence for recognition of epitopes on the extracellular face of the plasma membrane. The antigens recognised were not species or tissue specific. All of the antibodies raised were shown to recognise carbohydrate groups, a feature which has also been reported by Anderson *et al.* (1984). Production of monoclonal antibodies to carbohydrate groups may occur because these groups are immunologically dominant. This was first noted by Anderson *et al.* (1984), and was subsequently reported in several

other studies (for example, Evans *et al.* , 1988; McManus *et al.* , 1988; Key and Weiler, 1988). Immunodominance of carbohydrate compounds is also known in the animal kingdom (reviewed by Feizi and Childs, 1987). The usefulness of such antibodies may be diminished if they recognise carbohydrate residues that are a common component of many plant glycoproteins or carbohydrates, since such antibodies would not permit detection of species or determination-specific epitopes. The possibility of monoclonal antibodies being produced which recognised common epitopes was predicted by Galfré and Milstein (1981). Most of the monoclonal antibodies to carbohydrate epitopes produced by Norman *et al.* (1986) recognised antigens common to several unrelated species.

Monoclonal antibodies raised by Villanueva *et al.* (1986) to protoplasts derived from root cells of *Glycine max* L. were screened for binding to plasma membrane. It was shown that the antibodies recognised a high molecular mass polypeptide and they were used in the study of mobility of plasma membrane components after treatment with agglutinin. Total membrane fractions from *Vicia faba* L. guard cell protoplasts and plasma membranes from epidermis were used by Key and Weiler (1988) to raise monoclonal antibodies. These were characterised by immunofluorescence and one cell line was shown to secrete antibodies to a polypeptide which occured only on guard cell membranes, and hence was differentiation-specific. Another example of the use of monoclonal antibodies in the study of differentiation was reported by Evans *et al.* (1988). In this study mice were immunised with extracts from flowers of *Nicotiana tabacum* L. and were screened for cross reaction with extracts from various organs including leaves, sepals, petals, stamens and pistils. About half of the antibodies raised recognised carbohydrate epitopes, but some of these recognised antigens that were restricted to one type of cell or tissue. Antibodies which recognised a class of N-linked oligosaccharides from abscission tissue of *Sambucus nigra* L. were raised by McManus *et al.* (1988). It was shown that one of the cell lines recognised a range of plant enzymes (including horseradish peroxidase, which contains such oligosaccharide residues) and that an N-linked oligosaccharide inhibited

the binding of this antibody to horseradish peroxidase.

The examples given above indicate some of the uses and advantages of monoclonal antibodies in the study of plant cell membranes and enzymes. Further examples of the uses of monoclonal antibodies may be found in the collection of review articles edited by Wang (1986). The results presented in Appendix III and this chapter document the production of monoclonal antibodies to the calmodulin-stimulated, calcium pumping ATPase (CSCPA) of *Zea mays* and show the use to which such antibodies may be put.

6.2 RESULTS

The data presented in Appendix III shows the results obtained for production of monoclonal antibodies to the maize CSCPA. The CSCPA was purified by calmodulin-affinity chromatography (using a two hour column wash and buffers without PMSF or asolectin) and was dialysed into PBS. The dialysed sample (subsequently referred to as the 'antigen', containing 6-60 μg of protein by modified Lowry assay) was injected into female BALB/c mice. Initial screening of the antigenic response of the mice was made by ELISA with antigen bound to the wells of the plate. It was shown that the mice had produced a strong immune response to the antigen after two interperitoneal injections. One mouse was sacrificed and its splenocyte cells fused with myeloma cells of strain SP2/0-Ag-14. From this fusion, six hybridoma cell lines were produced which (on the basis of the first ELISA screening) appeared to secrete antibodies to the CSCPA. These cell lines were further cultured and supernatants screened again by ELISA. From this process, four of the cell lines (EA6, CC10, ID1 and FF7) were shown to secrete antibodies which recognised the antigen, and these were cloned by limiting dilution to yield monoclonal cell lines, the antibodies from which were used in subsequent experiments. The remaining two cell lines (DF5 and JF9) were shown to be unstable after further culture. Cell line DF5 was shown to be secreting antibody (of class IgM) but this did not cross react with the antigen. Cell line JF9 was shown to have ceased secreting antibodies; these two cell lines were not cloned by limiting dilution but were maintained for use as controls in some subsequent experiments. The monoclonal antibodies were tested for binding with carbohydrate epitopes by periodate treatment of antigen-coated wells; monoclonal antibodies FF7 and ID1 were shown to recognise carbohydrate epitopes, whilst monoclonal antibodies EA6 and CC10 probably recognised polypeptide epitopes. The class of the antibodies was determined by Ouchterlony double immunodiffusion; all antibodies were shown to be of IgM class. These antibodies were then used to further study some aspects of the CSCPA as described below.

6.2.1 Immunoblotting

i). Immuno-dot blotting

Preliminary experiments were carried out to determine suitable experimental conditions for detection of polypeptides recognised by the monoclonal antibodies after transfer to nitrocellulose membranes. In these early experiments, dots of antigen were applied in doubling dilutions to strips of nitrocellulose membranes. The membranes were incubated with monoclonal antibody-containing tissue culture supernatants. Antibodies binding to the antigen were detected with gold-conjugated secondary antibodies followed by silver enhancement, as described for immunoblotting (Chapter 2, section 2:4:1). The result obtained (data not shown) indicated that binding by monoclonal antibodies EA6, CC10, ID1 and FF7 could be detected using this method; the intensity of the dots in the controls (supernatants from cell lines DF5 and JF9, and tissues culture medium alone) was absent (or much fainter) than for dots on membranes incubated with monoclonal antibody supernatants. Binding of the monoclonal antibodies to calmodulin alone was also investigated by this technique; the results indicated that there was no recognition of calmodulin by any of the antibodies.

ii). Immunoblotting against microsomal proteins with the monoclonal antibodies.

Microsomal proteins were prepared in the presence and absence of PMSF and were electrophoresed as described (Chapter 2, sections 2:3:1 and 2) on neutral 7.5% SDS-polyacrylamide gels. Proteins were electrophoretically transfered to nitrocellulose membranes (Chapter 2, section 2:4:1) and incubated with hybridoma supernatants. Antibody binding was detected using gold conjugated secondary antibodies followed by silver enhancement. The results obtained are shown in Figure 6.1 (microsomes prepared in the absence of PMSF) and Figure 6.2 (microsomes prepared with PMSF). In Figure 6.1 it can be seen that interaction of monoclonal antibodies with microsomal polypeptides did not produce intensely staining bands. For microsomes prepared in the absence of PMSF,

monoclonal antibody EA6 (Figure 6.1, lane a) faintly cross reacted with two polypeptides of M_r about 62,000 and 51,000. Monoclonal antibody CC10 (lane b) showed faint cross reaction at 62,000 M_r. There was no evidence of cross reaction with monoclonal antibodies ID1 (lane c), with the control cell lines DF5 (lane d) and JF9 (lane e) or with tissue culture medium alone (lane h). Monoclonal antibody FF7 (lane f) showed a broad band of staining at high relative molecular mass between about 100,000 and 140,000 M_r. A similar pattern was also seen for polyclonal antiserum (lane g).

Immunoblotting of microsome samples prepared in the presence of PMSF showed slightly different patterns of cross reaction, as shown in Figure 6.2. Monoclonal antibody EA6 (lane a) showed relatively strong binding at about 51,000 M_r, and also bound to polypeptides at about 40,000 M_r and 69,000 M_r. Monoclonal antibody CC10 stained only faintly and the blot showed high background staining (this blot was silver enhanced for the same length of time as the others). At least three bands could be seen at M_r values of 30,000, 40,000 and 69,000. Monoclonal antibody ID1 (lane c) showed several bands of binding at M_r 40,000, 69,000 and 77,000. There was no binding of supernatants from cell lines DF5 (lane d) or JF9 (lane e), or with TCM alone (lane h). As was the case for the immunoblots of microsomes prepared without PMSF, monoclonal antibody FF7 (lane f) and polyclonal antiserum (lane g) showed diffuse regions of binding at high relative molecular mass, and also appeared to recognise a band which ran close to the dye front of the gel.

iii). Immunoblotting against purified CSCPA with monoclonal antibodies.

Samples of CSCPA were prepared as described (Chapter 2, section 2:2:3) in the presence or absence of PMSF. Electrophoresis (7.5% neutral SDS-polyacrylamide gels) and immunoblotting were carried out as described (Chapter 2, section 2:3, 2:4:1). It was found (data not shown) that, if purified CSCPA was divided into more than two parts prior to electrophoresis, insufficient protein was present on the nitrocellulose membranes for the antibodies to bind detectably. For this reason in subsequent experiments

only the potentially interesting monoclonal antibodies EA6 and CC10 were screened against purified CSCPA. With hybridoma supernatants of monoclonal antibodies EA6 and CC10, no binding could be detected with CSCPA purified in the absence of PMSF, even after several cycles of silver enhancement (Figure 6.3, lanes a and b). Immunoblotting against CSCPA purified in the presence of PMSF produced no staining with CC10 tissue culture supernatant (Figure 6.3, lane c), but monoclonal antibody EA6 bound to two bands in the purified CSCPA preparation at M_r 140,000 and 91,000 (Figure 6.3, lane d).

iv). Immunoblotting against erythrocyte ghosts with monoclonal antibodies.

Samples of erythrocyte ghost proteins (supplied by P. James, Eidgenössiche Technische Höchschule, Zürich) were electrophoresed on 7.5% SDS-polyacrylamide minigels, electrophoretically transferred to nitrocellulose membranes and then incubated with monoclonal antibody tissue culture supernatants. As shown in Figure 6.4 (lane a) monoclonal antibody EA6 recognised a polypeptide at approximately 205,000 M_r. No binding was present with the other antibodies or controls.

6.2.2 Effect of monoclonal antibodies on calmodulin-stimulated ATPase activity in microsomes

The assays were set up as described in Chapter 2, section 2:4:2; it was found in preliminary experiments that undiluted tissue culture medium alone apparently affected the extent of calmodulin-stimulation of ATPase activity. For this reason, monoclonal antibody-containing tissue culture supernatants and tissue culture medium control were diluted into Tris-buffered saline (TBS) before inclusion into the assay. A minimum dilution of 5,000-fold was required before the effects of TCM were overcome. It was necessary to correct the values obtained from the assays for the effect of TBS before calculation of the percentage effect of the monoclonal antibodies on calmodulin-stimulated ATPase activity. The calculation was made as below:

$$\frac{Activity\ in\ presence\ of\ antibody}{Activity\ in\ absence\ of\ antibody} \times 100$$

The results obtained are shown in Figure 6.5; monoclonal antibodies EA6 and CC10 appeared to inhibit a proportion of calmodulin-stimulated ATPase activity when diluted 5,000-fold, but did not inhibit activity at greater dilutions, nor did the control cell line JF9.

Figure 6.1: Immunoblotting of microsomes (prepared in the absence of PMSF) with monoclonal antibodies to maize CSCPA.

Electrophoresis on 7.5% SDS-polyacrylamide gel prior to transfer to nitrocellulose membranes.

Poly = polyclonal antiserum, 800-fold diluted.

TCM = tissue culture medium, with 10% foetal bovine serum.

Gel electrophoresis and electrophoretic transfer to nitrocellulose membranes carried out as described (Figures 4.6 and 4.13 respectively).
Nitrocellulose membranes were blocked in 5% BSA in Tris-buffered saline at room temperature. After washing, the membranes were incubated in undiluted hybridoma supernatants, with 1% normal goat serum added.
Use of the secondary gold conjugated antibodies and silver enhancement as described in Figure 4.13.

These methods were used in the subsequent figures (Figures 6.2-6.4).

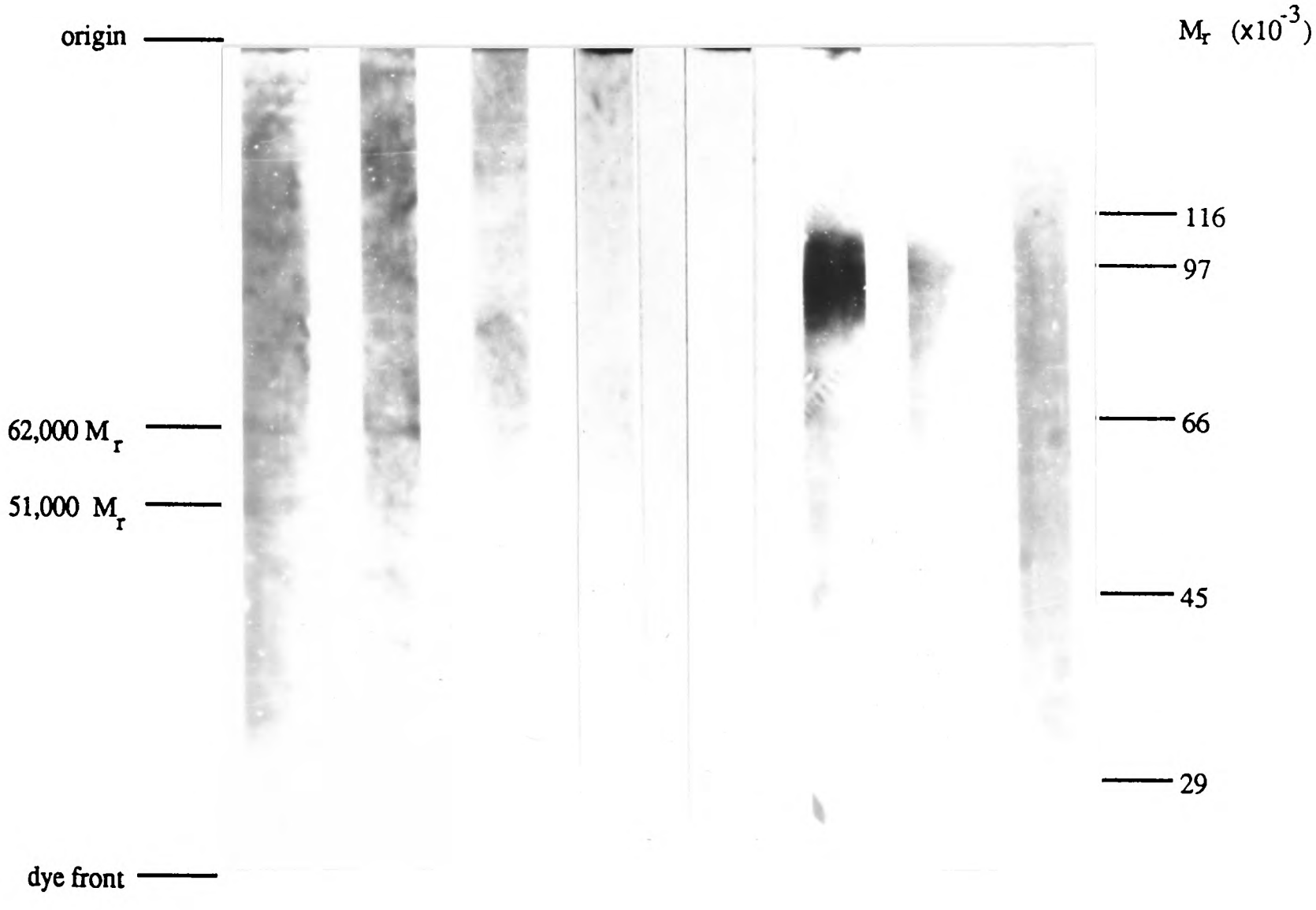

origin
62,000 M_r
51,000 M_r
dye front
M_r ($x10^{-3}$)
116
97
66
45
29
a
b
c
d
e
f
g
h
EA6
CC10
ID1
DF5
JF9
FF7
poly
TCM

Figure 6.2: Immunoblotting of microsomes (prepared in the presence of PMSF) with monoclonal antibodies to maize CSCPA.

Electrophoresis on 7.5% SDS-polyacrylamide gel prior to transfer to nitrocellulose membranes.

Poly = polyclonal antiserum, 800-fold diluted.

TCM = tissue culture medium, with 10% foetal bovine serum.

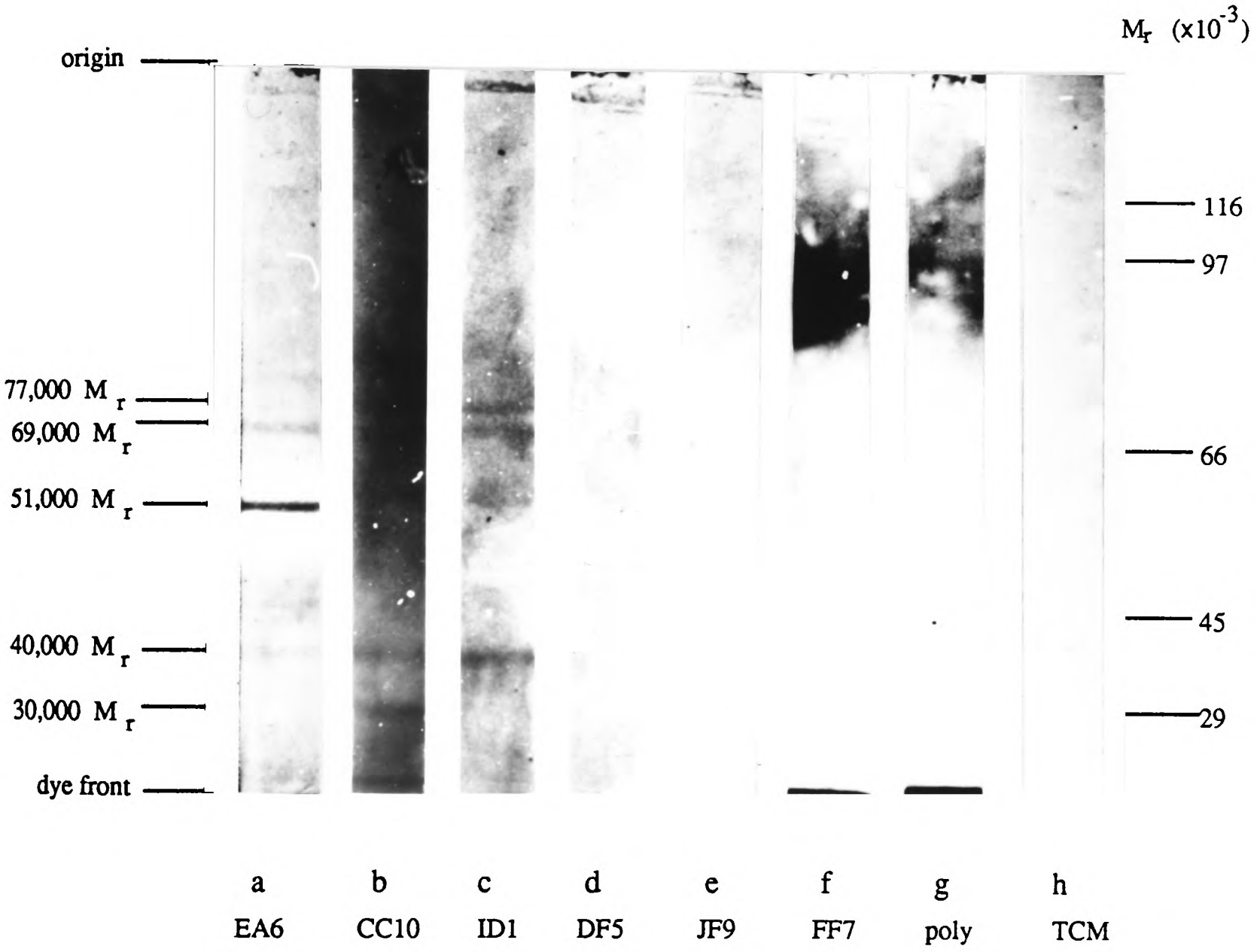

origin
77,000 M r
69,000 M r
51,000 M r
40,000 M r
30,000 M r
dye front
M r (x10-3)
116
97
66
45
29
a
EA6
b
CC10
c
ID1
d
DF5
e
JF9
f
FF7
g
poly
h
TCM

Figure 6.3: Immunoblotting of maize CSCPA (purified in the presence and absence of PMSF) with monoclonal antibodies.

Lane a: CSCPA (-PMSF) immunoblotted with EA6 hybridoma supernatant.

Lane b: as lane a, but with CC10.

Lane c: CSCPA (+PMSF) immunoblotted with EA6 hybridoma supernatant.

Lane d: as lane c, but with CC10.

Electrophoresis on 7.5% SDS-polyacrylamide gel prior to transfer to nitrocellulose membranes.

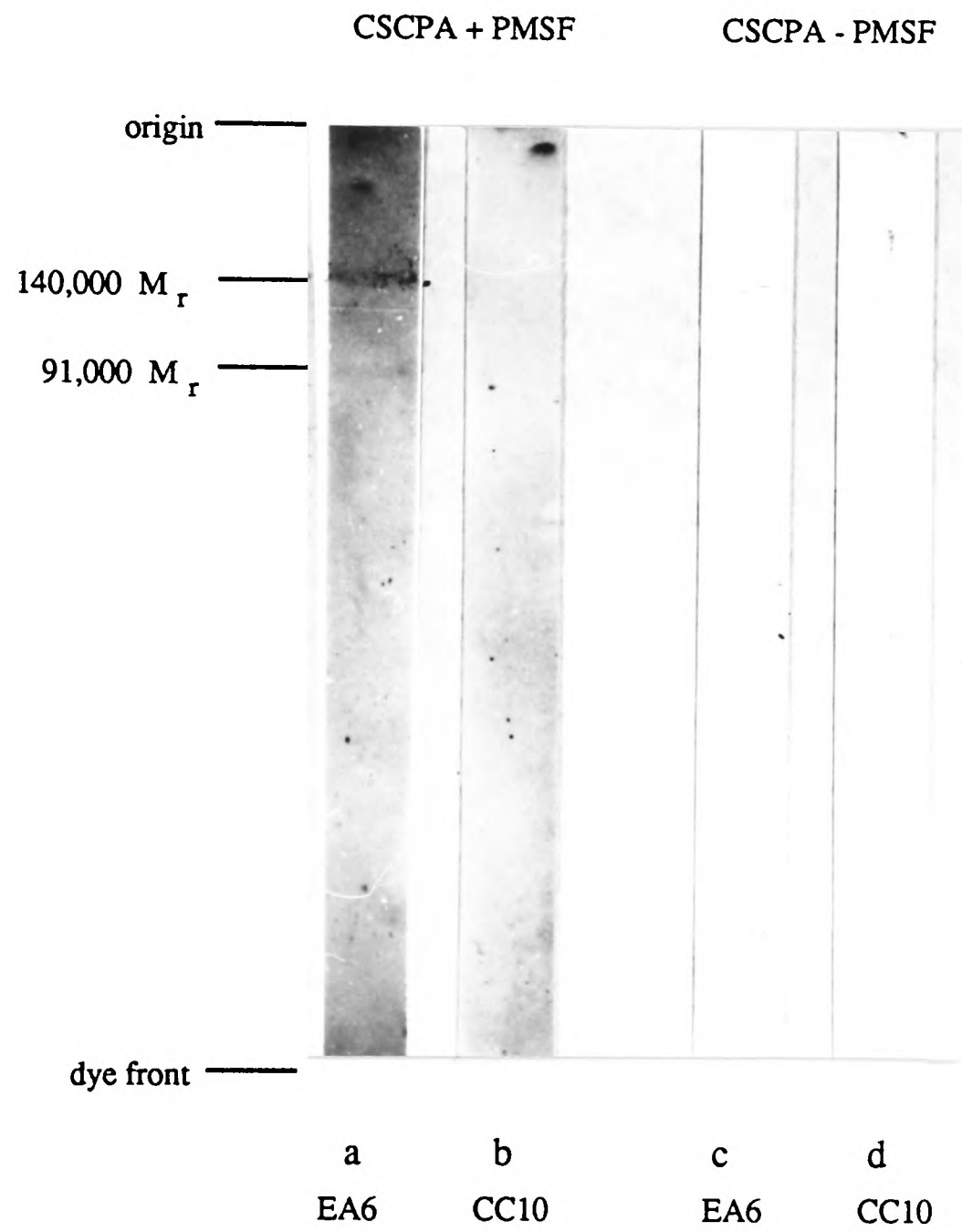

CSCPA + PMSF
CSCPA - PMSF
origin
140,000 M r
91,000 M r
dye front
a
EA6
b
CC10
c
EA6
d
CC10

Figure 6.4: Immunoblotting of erythrocyte ghost proteins with monoclonal antibodies to maize CSCPA.

Poly = polyclonal antiserum, diluted 800-fold.

TCM = tissue culture medium with 10% foetal bovine serum.

Electrophoresis on 7.5% SDS-polyacrylamide gel prior to transfer to nitrocellulose membranes.

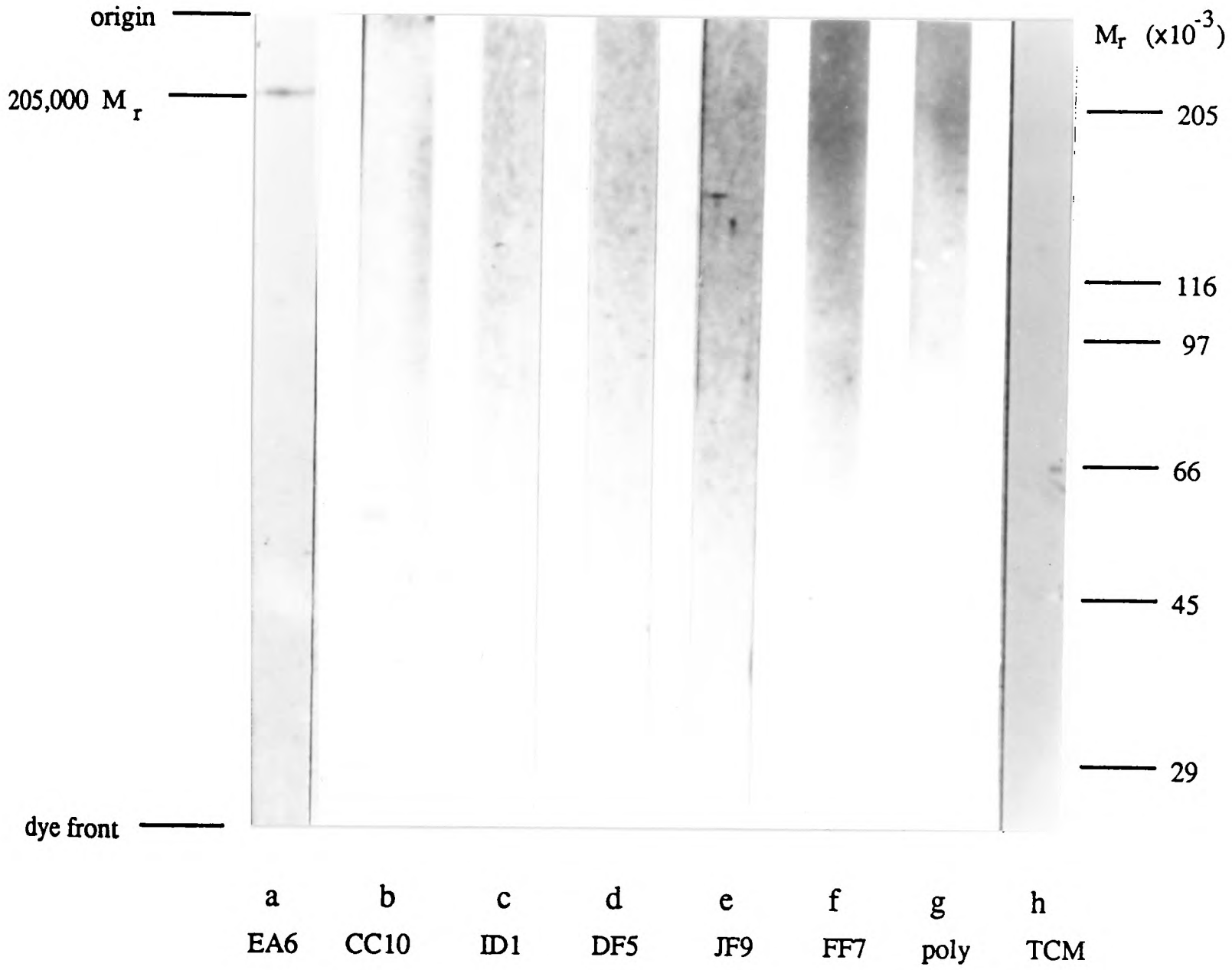

origin
205,000 M r
dye front
Mr (x10-3)
205
116
97
66
45
29
a
EA6
b
CC10
c
ID1
d
DF5
e
JF9
f
FF7
g
poly
h
TCM

Figure 6.5: Effect of monoclonal antibodies on calmodulin-stimulation of ATPase activity in microsomes.

Radioisotope ATPase assay; hybridoma supernatants diluted into TBS. Values are corrected for effects of TBS (see text) and are means of triplicate determinations.

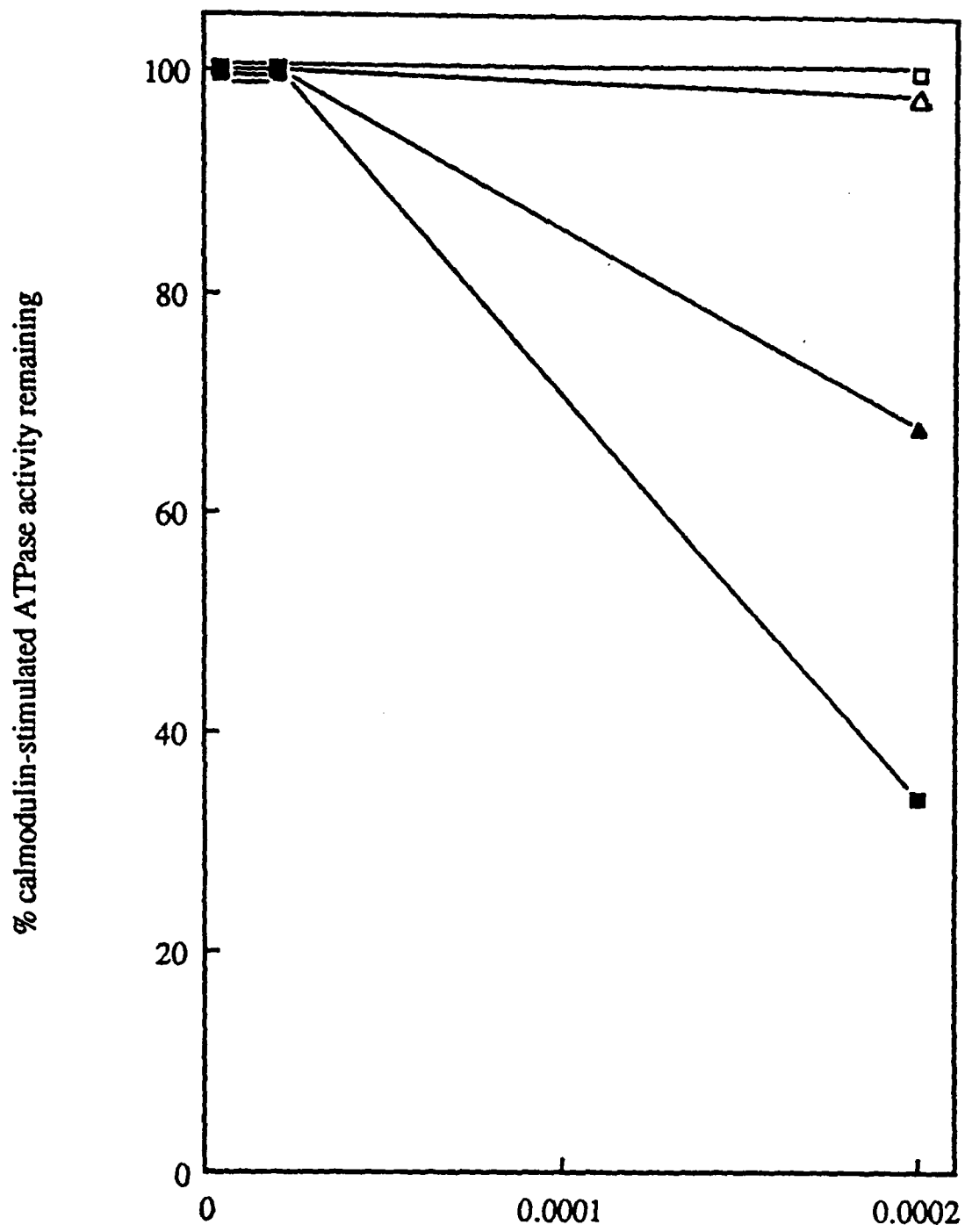
■ EA6

▲ CC10

□ FF7

△ JF9

The variation between the triplicates of these assays did not exceed plus or minus 5%


% calmodulin-stimulated ATPase activity remaining
100
80
60
40
20
0
0
0.0001
0.0002
Dilution of monoclonal antibody

6.3 DISCUSSION

6.3.1 Production of monoclonal antibodies

From the results presented in Appendix III, it was shown from the first screening procedure that six monoclonal antibodies were raised which apparently recognised the fraction purified by calmodulin-affinity chromatography and immobilised on ELISA plates. It was most likely that the CSCPA preparation used to inject the mice contained polypeptides of M_r 62,000, 66,000 and 72,000 (from SDS-polyacrylamide gel electrophoresis of the CSCPA purified in the absence of PMSF, Chapter 4, Figure 4.1). It was shown that this fraction contained calmodulin-stimulated ATPase activity (Chapter 4, Figure 4.5). It may have been that the intact CSCPA (of M_r 140,000) was present in this sample before electrophoresis, possibly in small amounts. It was of interest to know whether the dialysed sample of CSCPA (purified in the absence of PMSF) contained the intact CSCPA after binding to wells of ELISA plates. A method by which this could be investigated was to carry out the standard ELISA using the monospecific polyclonal antibody 97.1 as the primary antibody, as antibody 97.1 was shown to recognise only the intact CSCPA after immunoblotting (Chapter 4, Figure 4.13a). No binding of antibody 97.1 occured at any of the dilutions used. This suggested that the intact CSCPA was not bound to the ELISA wells and was probably not present in the fraction used to immunise the mice. There are alternative explanations for this result, including the intact CSCPA being present on the ELISA wells but in the wrong conformation for recognition by this antibody, or that the conformation of amino acid residues recognised by 97.1 was disrupted by binding of the CSCPA to the ELISA plate. The latter is considered unlikely, given that polyclonal antibody 97.1 recognised the 140,000 M_r intact CSCPA after immunoblotting.

Assays of polyclonal serum after the first immunisation of the mice (Figure III.2) showed a strong immune responce to the maize CSCPA. This was perhaps surprising, since cross reaction of anti-erythrocyte CSCPA polyclonal antibody 97.1 with the maize

CSCPA implied that there was homology between these two calcium pumps. It is usually expected that a protein which shows a high level of homology in the species from which it was extracted and the species into which it is injected will produce a weak immune response (reviewed in Harlow and Lane, 1988). However, it may be that polyclonal antibody 97.1 recognised one of the strongly homologous domains found in all P-type ion translocating ATPases sequenced to date whereas the strong immune response was to non-conserved regions. Alternatively it may be that the strong immune response arose as a result of the production of antibodies against some other component (which was not the CSCPA) in the fraction used to immunise the mice (see below for further discussion).

The fusion of splenocytes from mouse NE with myeloma cells yielded six cell lines that initially appeared to secrete antibodies to the CSCPA bound to ELISA wells. One possible reason why few hybridomas were formed may be that the antibodies produced in the polyclonal antiserum arose from the triggering of B lymphocytes without intervention by helper T cells. Such antibodies are known to arise from polymeric carbohydrate antigens (reviewed in Guttmann, 1981) which may have been a component of the sample used to immunise the mice. Antibodies produced in this way (instead of the normal T-helper cell dependent pathway) are known to be mainly of class IgM and also to produce no 'memory' in the B-cells such that these B-cells do not retain antigen-secreting capacity when fused with myeloma cells.

It was likely that cell lines DF5 and JF9, which appeared to secrete antibody to the CSCPA after the first screen of the supernatants from the hybridomas by ELISA may have arisen from non-fused or poorly stable cells which did not survive culture. The four remaining hybridomas were cloned by limiting dilution, a process which was entirely successful for cell lines FF7, ID1 and CC10, but which showed that cell line EA6 contained an element of genetic instability, because some of the cells from both cloning procedures appeared to lose antibody secreting capacity (Figures III.3, III.4). However,

this cell line was retained because preliminary assays (data not shown) indicated that supernatants from the cell line gave a strong response to the immunogen by ELISA. The supernatants used in the experiments shown in this chapter were those from cell lines showing strong cross reaction with the antigen after the second cloning process.

Periodate oxidation of the antigen bound to ELISA plates provided a simple test to determine whether the monoclonal antibodies recognised carbohydrate epitopes. Treatment of ELISA plates with periodate did not affect binding of CC10 and EA6, indicating that these monoclonal antibodies recognised polypeptides, whereas monoclonal antibodies ID1 and FF7 recognised carbohydrate groups. It is not known whether the maize CSCPA is glycosylated. In an early report of purification of the erythrocyte CSCPA (Penniston *et al.* , 1980) it was suggested that carbohydrate was associated with the CSCPA, but this was later suggested to be a result of contamination of the preparation by glycophorin (Graf *et al.* , 1982). In this report it was shown that very small amounts of carbohydrate were detectable when the gel was heavily loaded. It was not possible to stain gels of the purified maize CSCPA for carbohydrate content because of lack of a sufficiently sensitive carbohydrate stain. It remains a possibility that purification of the maize CSCPA by means of a two hour column wash without asolectin or PMSF did not remove some associated carbohydrate groups. If some carbohydrate groups were present in the immunogen, it is likely that these would form the immunologically dominant components, and hence many of the cell lines produced would secrete antibodies to carbohydrates, as suggested by Anderson *et al.* (1984). It was therefore possible that the monoclonal antibodies produced which recognised carbohydrate groups were not recognising components of the CSCPA. This may well be so for antibody FF7, but lines of evidence which indicate that antibody ID1 may recognise components of the CSCPA (and hence that the maize CSCPA may be glycosylated or tightly associated with some carbohydrates) are discussed below. As a result of this it was considered that monoclonal antibodies EA6 and CC10 were likely to be of most interest for studying

WITH PMSF			WITHOUT PMSF	
Coomassie-stained gel of CSCPA	Immunoblots		Immunoblots vs. microsomes	Coomassie-stained gel of CSCPA
	vs. CSCPA	vs. microsomes		
[a] 140 —	140 —			
91 —	91 —			
77 —				
69 —		69 —	69 —		72 —
					66 —
			62 —	62 —	62 —
51 —		51 —	51 —		
40 —		40 —	40 —		
			30 —		
Antibody	**EA6**	**EA6**	**CC10**	**EA6**	**CC10**	

Summary of results of immunoblotting *vs.* various fractions with monoclonal antibodies EA6 and CC10 (see text opposite for details).

[a] values are relative molecular mass ($x10^{-3}$)

the possible polypeptide subunits of the CSCPA.

6.3.2 Use of the monoclonal antibodies

The antibodies were first screened by immuno-dot blotting to ensure that the detection system chosen (gold-conjugated anti-mouse polyvalent secondary antibodies followed by silver enhancement) permitted antibody binding to the antigen bound to nitrocellulose to be detected. Dot blots were also carried out against calmodulin bound to the nitrocellulose membranes, the results of which showed no cross reaction (data not shown). This result was of importance because the calmodulin-binding domain contained in the C-terminal region of the erythrocyte CSCPA resembles calmodulin itself (Brandt *et al.* , 1988) and it was suggested (Professor E. Carafoli, pers. comm.) that some of the antibodies raised to the erythrocyte CSCPA recognised calmodulin as well as the CSCPA.

i). Immunoblotting.

The results of gel electrophoresis of the CSCPA purified in the presence and absence of PMSF, and of immunoblotting with the monoclonal antibodies EA6 and CC10 against CSCPA (with PMSF) and microsomes (with and without PMSF) are summarised diagrammatically opposite:

a) Immunoblotting *vs* CSCPA purified in the presence of PMSF.

Antibody EA6 recognised intact CSCPA at 140,000 M_r (Figure 6.3) together with a second polypeptide at 91,000 M_r which was likely to be a proteolysis product of the intact CSCPA (see below).

b) Immunoblotting *vs* CSCPA purified in the absence of PMSF.

Monoclonal antibodies EA6 and CC10 did not recognise any polypeptides in the CSCPA fraction purified in the absence of PMSF after electrophoresis and immunoblotting (Figure 6.3). This result may be a consequence of insufficient protein being present on the nitrocellulose membrane. The fact that monoclonal antibody EA6 recognised 2

ng of CSCPA on ELISA plates when diluted 2048-fold (data not shown) suggested that the amount of protein present on the immunoblots was perhaps less likely to be a limiting factor than the possibility that the epitopes recognised by monoclonal antibodies EA6 and CC10 were not exposed in the CSCPA purified in the absence of PMSF and transfered to nitrocellulose.

c) Immunoblotting *vs* microsomes prepared in the absence of PMSF.

Immunoblotting of the anti-maize CSCPA monoclonal antibodies against microsome proteins extracted without PMSF (Figure 6.1) showed that CC10, EA6 and FF7 recognised various bands. Antibody EA6 recognised polypeptides at 62,000 and 51,000 M_r. It may be suggested that the 62,000 M_r polypeptide recognised by EA6 was the same as that shown in Chapter 4, Figure 4.1, after purification of the CSCPA without PMSF. On the gel of the CSCPA purified without PMSF, no band at 51,000 M_r was visible, but it is possible that this polypeptide was present in amounts too low to be detected by the staining method used and may correspond to that at 51,000 M_r shown in gels of the CSCPA purified in the presence of PMSF (Chapter 4, Figure 4.12). Antibody CC10 also recognised a polypeptide at 62,000 M_r. The pattern of staining seen by polyclonal serum and monoclonal antibody FF7 (both of which recognised carbohydrate epitopes) was indicative of recognition of high M_r glycoproteins, a common component of plant extracts. It was possible that carbohydrate components which may have been present in the CSCPA preparation used to immunise the mice were also present in association with proteins of high M_r in the microsome fraction.

d) Immunoblotting *vs* microsomes prepared in the presence of PMSF.

The pattern of polypeptides recognised by the monoclonal antibodies in microsomes extracted in the presence of PMSF was slightly different from that described above. Monoclonal antibody EA6 recognised a polypeptide at 40,000 M_r and at about 69,000 M_r. These values correlate with those obtained after electrophoresis of the CSCPA purified in the presence of PMSF (Chapter 4, Figure 4.12). Monoclonal antibody CC10

recognised a polypeptide at 40,000 M_r, corresponding with a polypeptide on the gel of the purified CSCPA, and also recognised a polypeptide at 30,000 M_r. On SDS polyacrylamide gels of the CSCPA purified in the presence of PMSF, a polypeptide of this relative molecular mass was not visible, but a polypeptide of this M_r value was present on acid gels of purified CSCPA (Figure 5.8). Therefore it was possible that the polypeptide at 30,000 M_r recognised by CC10 was a proteolysis product of the CSCPA. It was interesting that monoclonal antibody ID1 recognised components only in microsomes prepared in the presence of PMSF, showing cross reaction at M_r 40,000, 69,000 and 77,000. These values again correspond with those of polypeptides from the fraction purified in the presence of PMSF. The identification of ID1 as an antibody which recognised carbohydrate epitopes suggests that these polypeptides were associated with carbohydrate components. If it is assumed that these polypeptides are proteolysis products of the CSCPA, then this result suggests that at least some of the proteolysis products are glycosylated or associated with carbohydrate components. As indicated above, it is considered that the erythrocyte CSCPA is not glycosylated (Graf *et al.* , 1982); as yet no information is available concerning whether the maize CSCPA is similar to that of the erythrocyte in this respect, it may be argued that the bands recognised by antibody ID1 on the immunoblot against microsomes prepared in the presence of PMSF are not related to the CSCPA. However, the fact that antibody EA6 (which recognised the intact CSCPA) recognised some of the same bands as ID1 (at 40,000 and 69,000 M_r) in the microsome preparation prepared in the presence of PMSF implies that these components are related to the CSCPA and are likely to be proteolysis products. Monoclonal antibody FF7 and polyclonal antiserum showed the same pattern of cross reaction against microsomes extracted with PMSF and for microsomes extracted without PMSF.

e) Immunoblotting *vs* erythrocyte ghosts.

Monoclonal antibody EA6 appeared to recognise a polypeptide of about 205,000

M_r in a sample of erythrocyte ghost protein (Figure 6.4). It has been reported that the erythrocyte CSCPA forms a dimer of approximately 205,000 M_r (Niggli *et al.* , 1979b), and it was possible that EA6 recognised the dimer of the erythrocyte CSCPA, inadequate dissociation to monomer having been effected prior to electrophoresis.

f) Evidence concerning the identity of lower M_r polypeptides recognised by immunoblotting.

Whilst the 140,000 M_r polypeptide recognised by antibody EA6 is the intact CSCPA the identity of lower M_r polypeptides recognised by the antibodies remains in question. It seems likely that many of them will be proteolysis products of the intact CSCPA. The fact that their presence depends on the mixture of protease inhibitors present indicates that their M_r is greatly affected by proteolysis. Further evidence depends on the assumed specificity of the monoclonal antibodies used. Monoclonal antibodies only recognise a small region of the antigen and, if this region is also present in other polypeptides, lack of specificity will result; however, if the epitope is unique to the antigen, the antibody will only recognise the antigen itself or proteolysis products of it (Lane and Koprowski, 1982). If the latter is the case, most convincing evidence for the identity of the lower M_r polypeptides as proteolysis products of the intact CSCPA comes from antibody EA6, which recognised the intact antigen. EA6 recognised polypeptides of 40,000 and 69,000 M_r in microsomes purified in the presence of PMSF, 62,000 and 51,000 M_r in microsomes purified in the absence of PMSF, and 91,000 and 140,000 M_r in the CSCPA preparation purified in the presence of PMSF. Antibody CC10 also recognised the 62,000 M_r polypeptide, together with a further 40,000 M_r polypeptide (see summary above). All of these bands are visible in gels of CSCPA purified in the presence or absence of PMSF (see Chapter 4 for details) and many coincide closely with the known major trypsin digest pattern of the erythrocyte CSCPA (91,000, 81-76,000, 50,000, and 42,000 M_r, Carafoli and Zurini, 1982). It should be noted at this point that recognition by the antibody of a transferred polypeptide is also dependent on that

polypeptide presenting sufficient antigenic site in correct configuration for binding. Thus it should not be surprising that not all monoclonal antibodies recognise all the lower M_r bands in the preparations. This must be assumed to be the case for polyclonal antibody 97.1 which only recognises intact purified maize CSCPA (Chapter 4). Similarly, lack of recognotion of the 140,000 M_r polypeptide in microsomal extracts is likely to be a result of either low abundance or incorrect antigenic configuration of the CSCPA in that preparation. It was also possible that other proteins in the microsome fraction may have interacted with the CSCPA such that it was not recognised by antibody EA6. If this is so, then it appears that these sites became available for recognition when the CSCPA was purified, and were also available in some of the proteolysis products in the microsome fraction.

ii). Effects of antibodies on ATP hydrolysis

Demonstration of the effect of the monoclonal antibodies on calmodulin stimulation of ATPase activity in microsomes was not straightfoward. The first experiments carried out to investigate antibody effects on ATPase activity indicated that tissue culture medium (included as a control) inhibited calmodulin stimulated ATPase activity when incorporated undiluted into the assay. Experiments in which hybridoma supernatants and TCM supernatants were diluted into Tris-buffered saline (TBS) before addition to the assay showed that tissue culture medium diluted 5,000-fold did not affect calmodulin stimulation of ATPase activity, whilst monoclonal antibodies EA6 and CC10 at this dilution inhibited calmodulin stimulation (Figure 6.5). Monoclonal antibody FF7 did not inhibit calmodulin stimulated ATPase activity at this dilution, nor did the control cell line JF9; none of the antibodies inhibited activity at dilutions greater than 5,000-fold. In experiments where the monoclonal antibodies were diluted into TBS it was necessary to adjust the data for the effect of TBS, which stimulated basal and calmodulin stimulated ATPase activity. Reasons for the effect of TBS were not clear, but occured reproducibly.

The erythrocyte CSCPA has a structure such that the ATP binding site, the site where the phosphorylated intermediate is formed and the calmodulin binding domain are exposed from the membrane (see Chapter 7, Figure 7.1). It may be that antibodies EA6 and CC10 recognised regions at or near equivalent domains in the maize CSCPA, and so inhibited calmodulin-stimulated ATPase activity. Since these antibodies were of class IgM it was also possible that the apparent inhibition of calmodulin-stimulated ATPase activity by antibodies EA6 and CC10 was a result of the antibodies binding to sites distinct from the active site, calmodulin-binding site or phosphorylation site, inhibition of activity being brought about by steric hinderance by the pentameric IgM complexes.

It is interesting to compare these results with those obtained in some other studies where the effect of antibodies on plasma membrane ATPases has been investigated. No reports exist concerning production of monoclonal or polyclonal antibodies to plant calcium pumping ATPases of any membrane location. Monoclonal antibodies have been produced to the erythrocyte CSCPA, as reported by Borke *et al.* (1987). In this study, monoclonal antibodies were raised to purified erythrocyte CSCPA; however, it was not made clear whether the mice were immunised only with intact CSCPA, or whether it was established if intact CSCPA was bound to the wells of the ELISA plates. The initial screening of the hybridoma supernatants in this study was designed such that only antibodies of class IgG were selected. Immunoblotting showed that three of the monoclonal antibodies recognised the intact CSCPA at 138,000 M_r and also recognised a polypeptide at about 205,000 M_r, which represented the dimeric form of the CSCPA. The effects of these three antibodies on ATPase activity in the presence and absence of calmodulin were evaluated, and it was shown that two of these stimulated both basal and calmodulin stimulated ATPase activity. This result is in contrast to those obtained in this thesis, where it was shown that none of the anti-maize CSCPA antibodies stimulated basal ATPase activity to a greater extent than that in control assays with

calmodulin but no antibody. It was also shown by Borke *et al.* (1987) that some of the monoclonal antibodies produced cross reacted with plasma membranes in tissue sections from a variety of sources, whilst the other antibodies did not cross react. The report by Caride *et al.* (1988) demonstrated that it was possible to raise monoclonal antibodies against the extracellular face of the erythrocyte calcium pump. It has been shown (for references and further details, see Chapter 7) that most of the hydrophilic regions of the erythrocyte CSCPA are located on the cytoplasmic face of the plasma membrane, and that only a small proportion of the CSCPA is exposed from the extracellular face. In the study by Caride *et al.* (1988), monoclonal antibodies were raised as described by Borke *et al.* (1987). The tissue culture supernatants were first screened by ELISA with purified CSCPA bound to the plate walls. The secondary screen carried out selected for antibodies that bound to intact red blood cells, antibodies which reacted positively in both assays being those which recognised the extracellular face of the CSCPA. One cell line was selected and cloned after these assays. This antibody was immunoblotted against purified erythrocyte CSCPA and against erythrocyte membranes. This antibody recognised more than one polypeptide in the purified fraction, and was capable of recognising the intact CSCPA in the membrane fraction. It was used to precipitate antigens from intact red cells and the precipitated components were immunoblotted against an antibody raised by Borke *et al.* (1987). These experiments confirmed that the antibody produced by Caride *et al.* (1988) recognised the extracellular face of the CSCPA. It was shown that this monoclonal antibody had no effect on ATPase activity of the purified fraction. It also had no effect on calcium transport in resealed red cell ghosts. Its lack of effect on ATPase and calcium transport activity of the CSCPA was perhaps not surprising, given its proposed binding to the extracellular face of the CSCPA, and the fact that the ATP-binding site and calcium binding sites of the CSCPA are located on the cytoplasmic face (see Chapter 7 for details and references). Intact red cells were treated with various enzymes to investigate the nature of the epitope recognised by

the antibody. Enzymes which were specific for carbohydrate groups had no effect on binding of the antibody to intact red cells. Various proteases caused a reduction in the extent of antibody binding, indicating that the epitope recognised was a polypeptide. It was also stated that this epitope was not recognised by any of the polyclonal antibodies which had been produced to the erythrocyte CSCPA (see below for further details). The suggestion was made that a possible reason for the low percentage of antibodies raised to the extracellular face of the CSCPA was because this face was not recognised by the immune system of the mouse. This makes the assumption that the amino acid sequences of the extracellular domains of CSCPAs from different mammalian species are similar. As yet this suggestion cannot be verified because only the CSCPA from human erythrocytes has been sequenced (Verma *et al.* , 1988).

Polyclonal antibodies have been raised to purified erythrocyte CSCPA by Verma *et al.* (1982). The antiserum produced was used in competitive radioimmunoassay experiments where it was shown that the erythrocyte CSCPA showed immunological similarity to CSCPAs derived from other cell surface (plasma membrane) fractions. However this antiserum did not react with calcium pumping ATPases extracted from sarcoplasmic reticulum. Such ATPases are not regulated by calmodulin and have a relative molecular mass of 100,000, making them distinct from the CSCPA of the plasma membrane (Carafoli, 1987). The effect of the antiserum on basal and calmodulin-stimulated ATPase activity and calcium transport was evaluated; the antibodies inhibited activity in the presence and absence of calmodulin. The data was calculated as below:

$$\frac{Activity\,with\,immune\,IgG}{Activity\,with\,non-immune\,IgG} \times 100$$

The way in which the data was presented did not make it possible to distinguish whether the effect of the antiserum was on basal activity alone or on basal and calmodulin-stimulated activity. However, it was shown that antibodies from pre-immune serum did

not affect activity of the CSCPA and that the antiserum had no effect on calmodulin-binding to erythrocyte membranes. This work was extended in the study by Verma *et al.* (1984) in which polyclonal antibodies raised against the intact erythrocyte CSCPA were affinity purified. It was shown that the purified polyclonal antibodies inhibited Ca^{2+}-ATPase activity in purified CSCPA and erythrocyte ghost fractions, and also inhibited Mg^{2+}-ATPase activity found in erythrocyte ghosts. The antibodies had no effect on nitrophenyl phosphatase activity of erythrocyte membranes, and stimulated Na^{+}-K^{+}-ATPase activity of red cell ghosts. The antibodies were shown to inhibit Ca^{2+}-ATPase activity only from the cytoplasmic face of the erythrocyte membrane; they had no effect on Ca^{2+}-ATPase activity of the sealed red cell vesicles when present in the external medium. Gietzen and Kolandt (1985) raised polyclonal antibodies to human erythrocyte CSCPA. They demonstrated that these antibodies inhibited only the calmodulin-stimulated ATPase activity, having no effect on basal ATPase activity or on calcium transport. It was suggested that at least some of the antibodies in the antiserum recognised the calmodulin-binding site of the CSCPA. The antiserum had no effect on ATPase activity of sarcoplasmic reticulum calcium-pumping ATPase, and did not inhibit calmodulin-stimulated phosphodiesterase activity.

No reports exist concerning production of antibodies to plant calcium pumping ATPases. However, there is one study in which monoclonal antibodies which affected activity of the plasma membrane proton pumping ATPase were produced. Chin (1982) immunised mice with protoplasts from *Hordeum vulgare* L. The antibody-containing tissue culture supernatants produced were initially screened for cross reaction with plasma membrane proteins bound to nitrocellulose membranes. Cell lines which showed positive reaction were cloned and assayed for effect on pH 6.5 (K^{+}+Mg^{2+})-ATPase activity of a plasma membrane enriched fraction from the protoplasts. This activity is associated with the plasma membrane proton pumping ATPase, and is commonly used as a biochemical marker for plasma membrane. Several fusions were carried out, which

generated a total of five cloned cell lines producing antibody that affected (K^++Mg^{2+})-ATPase activity. Four of these were inhibitory and one stimulatory. It was suggested that these monoclonal antibodies could be of use in studying the proton-pumping ATPase and in the identification of plasma membrane fractions.

The data presented in this chapter has shown that a monoclonal antibody (EA6) which recognised intact purified CSCPA had been raised. Although no antibody which recognised intact CSCPA prior to purification was obtained, several antibodies were raised which recognised likely proteolysis products in both purified and microsomal extracts. Two of the antibodies appeared to inhibit calmodulin-stimulated ATPase activity of microsomes. Demonstration of the effects of purified antibodies on calmodulin-stimulated calcium transport in microsomes were found not to be straightfoward (data not shown), which was most likely to be caused by the antibodies being of class IgM. It is difficult to construct antibody-affinity columns with antibodies of class IgM; preliminary attempts made during the course of this study were not successful (data not shown). Production of monoclonal antibodies of class IgM has been reported in several studies where plant extracts have been used as immunogens (for example, Brewin *et al.* , 1985; Bradley *et al.* , 1988).

Several suggestions may be made concerning possible future studies on the production of monoclonal antibodies to maize CSCPA. One approach which could be tried with antibody EA6 produced in this study would be to attempt to isolate clones that had switched to secreting antibody of class IgG, as described by Harlow and Lane (1988). If subsequent fusions were attempted it would be advantageous to use an anti-mouse IgG horseradish peroxidase conjugate in the initial screening ELISAs so that antibodies of class IgM were not detected. The production of IgG antibodies would permit the construction of antibody affinity columns and would also facilitate immunostaining of tissue sections at the light and electron microscope level. It may be that IgG antibodies

would also have more straightfoward effects on activity of the CSCPA, allowing calcium transport to be studied by this means as well as ATPase activity. As a means of studying the proteolysis pattern of the CSCPA, bands cut from the gel could be used as the immunogens; the antibodies so produced could be used in conjunction with limited proteolysis techniques for peptide mapping (Cleveland, 1983).

Chapter 7

GENERAL DISCUSSION

The objective of the work carried in this thesis was to further characterise the calmodulin-stimulated calcium transport and ATPase activities associated with maize coleoptile microsomal membranes described by Dieter and Marmé (1980a,d, 1981b). It was suggested by Dieter and Marmé (1980d, 1981b) that these calmodulin-stimulated activities represented the action of a calmodulin-stimulated, calcium pumping ATPase (CSCPA) of similar properties to that found in mammalian erythrocyte cells, and therefore that plant cells contain this mechanism for regulating ${}_p[Ca]_{cyt}$.. The results obtained in this study have provided further evidence which indicates that this suggestion may well be true.

Inorganic phosphate release and ATP-dependent calcium transport activities in a microsomal fraction from *Zea mays* L. coleoptiles were characterised with respect to calmodulin-stimulation (Chapter 3 and Appendix I respectively). It was found that the calmodulin concentration range over which stimulation of these activities occurred was comparable in both cases. In addition the shape of the calmodulin-stimulation curves (that is, stimulation by calmodulin over 'basal' activity in the absence of calmodulin) was similar for ATP-dependent calcium transport activity and for Pi release activity (as measured by a spectrophotometric assay, see Chapter 3, section 3:3:1). Possible reasons

for differing levels of basal activity in the two assays have been discussed elsewhere (Chapter 3, section 3:3:2). The similar pattern of calmodulin-stimulation for these two assays suggests that they are features of the same system. It is possible that the Pi release measured in the spectrophotometric assay may have been the result of the activity of non-specific acid phosphatases, which were only active in the presence of ATP. Such enzymes may contribute to the basal Pi release measured, but no reports exist in which non-specific phosphatases have been shown to be stimulated by calmodulin, and hence the calmodulin-stimulated component of the Pi release activity measured was unlikely to be a result of such enzymes. The calmodulin-stimulated Pi release did not arise from action of a non-specific nucleotide triphosphate hydrolysing system, as shown from the high nucleotide specificity of this activity (Chapter 3, section 3:2:1). These data indicate that calmodulin-stimulated Pi release was most likely to have occurred as a result of activity of a calmodulin-stimulated ATPase, this being corroborated by subsequent data (see below).

The effect of sodium orthovanadate on calmodulin-stimulated microsomal ATPase activity was investigated, and it was shown (Chapter 3, section 3:2:1) that orthovanadate inhibited the calmodulin-stimulated component of Pi release with an I_{50} of 20 μM. Studies on the membrane-bound CSCPA of erythrocyte have indicated that orthovanadate is inhibitory at similar concentrations (Barrabin *et al.* , 1980; Bond and Hudgins, 1980); this property is considered to be a characteristic of P-type ATPases (see Chapter 1). Orthovanadate is not known to inhibit activity of non-specific phosphatases or other nucleotide triphosphate hydrolysing systems.*

Further evidence indicating that true calmodulin-stimulated ATPase activity was present in the microsomal fraction was gained from use of a radioisotope assay, in which only ^{32}P released from [$\gamma^{32}P$]-ATP was measured. Calmodulin-stimulation of ^{32}P release in this assay showed a similar shape to that of calcium transport and Pi release up to a concentration of about 0.1 μM (Chapter 3, section 3:2:2). Reasons for apparent decline

* Vanadate does not inhibit non-specific phosphatases or other nucleotide triphosphate hydrolysing systems when used at concentrations less than 100 μM; however at concentrations greater than this, non-specific effects are likely to occur.

in calmodulin-stimulation at higher calmodulin concentrations have been discussed elsewhere (Chapter 3, section 3:3:2). Together these lines of evidence extend and confirm previous reports (for references see Chapter 1) demonstrating calmodulin-stimulated calcium transport and ATPase activity in microsomes. The evidence presented for the effect of orthovanadate and nucleotide specificity further suggest a comparison with the erythrocyte CSCPA. The procedure used to prepare the microsomal fraction included a step in which a 'crude microsomal' pellet from centrifugation at 48,000xg was resuspended and centrifuged again, to produce a 'washed microsomal' fraction which was used in all the assays. Demonstration of calmodulin-stimulation of ATPase activity and ATP-dependent calcium transport in the washed microsomal fraction provided evidence that these activities were associated with a membrane-bound system, rather than a soluble one.

Calmodulin-affinity chromatography was used as a means of purifying this calmodulin-stimulated activity further. Membrane proteins from the washed microsome fraction were solubilised using Triton X-100 prior to affinity chromatography. It was shown that calmodulin-stimulated ATPase activity (measured using the spectrophotometric assay, data not presented) was present in the Triton X-100 solubilised fraction. This further suggests that the calmodulin-stimulated ATPase activity arose from activity of a membrane protein, and not from a cytoplasmic or soluble protein.

Calmodulin-binding proteins were eluted from the calmodulin-affinity column with a buffer containing EGTA. After calmodulin affinity chromatography in the absence of protease inhibitors or asolectin (see below) the fraction eluted by EGTA was shown to contain calmodulin-stimulated ATPase activity as measured by the radioisotope assay (Chapter 4, section 4:2:1). The dose-response curve for addition of calmodulin to this fraction (and to fractions purified using column buffers containing asolectin, Chapter 4, section 4:2:1) was similar to that for calmodulin stimulation of ATPase activity and ATP-dependent calcium transport in the microsomal fraction. This suggested that

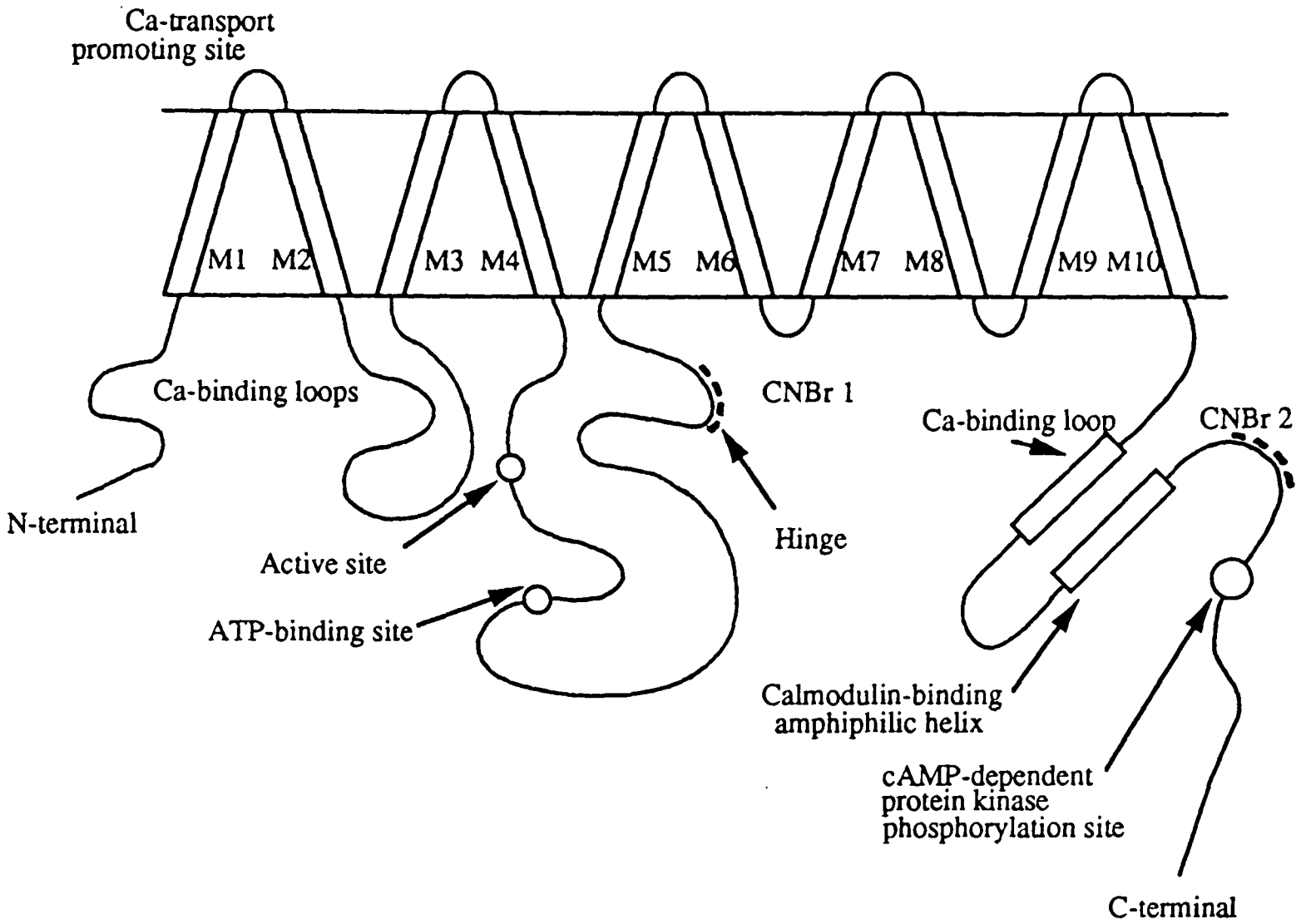
activity of the same system was being measured in the purified and microsomal fractions. It seems likely that calmodulin-stimulated activity in the EGTA-eluted fraction from the affinity column was the result of direct activation by calmodulin and was not, for example, mediated by a soluble calmodulin-dependent phosphokinase.

It was found that the relative molecular mass (M_r) values of the polypeptides present in the EGTA-eluted fraction from affinity chromatography were strongly dependent on the presence of the protease inhibitor PMSF during extraction and purification, PMSF and chymostatin also being included during electrophoresis. In the absence of these protease inhibitors, polypeptides of 62,000, 66,000 and 72,000 M_r were present, and calmodulin-stimulated ATPase activity was shown in the fraction. Assuming that degradation did not take place during electrophoresis this suggests these polypeptides contained domains for hydrolysis of ATP and for calmodulin-stimulation of activity. The M_r of the intact erythrocyte CSCPA is 134,683 (Verma *et al.* , 1988) and hence this data suggested either that the polypeptides mediating calmodulin-stimulated ATPase activity in maize were smaller than those of erythrocytes or that proteolysis had occurred. This latter possibility appears to be most likely in the light of subsequent data (see below). After extraction, purification and electrophoresis in the presence of protease inhibitors, a polypeptide of approximately 140,000 M_r was present on neutral and acidic SDS-polyacrylamide gels (Chapter 4, section 4:2:2, Chapter 5, section 5:2:3), together with some other polypeptides of lower M_r (see below and Chapter 4, section 4:3:2). The similarity in size to the intact erythrocyte CSCPA strengthened the likelihood that maize cells contain a system similar to the CSCPA of erythrocytes. This was further corroborated by demonstration of immunological homology between the erythrocyte CSCPA and the polypeptide of 140,000 M_r in the EGTA-eluted fraction, which bound an affinity purified monospecific antibody raised to the erythrocyte CSCPA (polyclonal antibody 97.1, which recognises only the intact form of the CSCPA, Chapter 4, section 4:2:2). This data provides the strongest evidence available to date

indicating that maize cells contain a system similar to the CSCPA of erythrocytes.

Binding of antibody 97.1 provided indirect evidence which suggested that the location of the maize CSCPA was at the plasma membrane, as antibody 97.1 (see Chapter 4, section 4:3:2) bound only to calcium-pumping ATPases located at the limiting membrane of mammalian cells. In addition, in animal cells there is no evidence for calmodulin-stimulation of calcium-pumping ATPases located in membranes other than the limiting membrane and hence demonstration of calmodulin stimulation in the purified maize preparation may be taken as indirect evidence for a probable plasma membrane location of the maize CSCPA. Additional evidence is found in the report of Robinson *et al.* (1988) in which a calmodulin-stimulated ATPase was shown to be localised in a well characterised plasma membrane fraction, and showed very similar properties to the system described here. Thus it seems likely that maize cells contain a calmodulin-stimulated ATPase activity that is similar to the CSCPA of erythrocytes and which is most probably located at the plasma membrane. The limited amount of amino acid sequence data which was obtained in collaboration with Professor E. Carafoli lends further weight to this hypothesis (see Appendix II, Chapter 4, section 4:3:2 and Figure 7.1). Studies are currently in progress in Oxford and Zürich to elucidate the full cDNA sequence of the maize CSCPA, which would provide a means for detailed comparison between maize and erythrocyte CSCPA.

In order to investigate the maize CSCPA further a series of experiments were carried out (Chapter 5) to examine its reaction mechanism in both purified and microsomal fractions. A rapidly turning over, calcium-dependent and hydroxlamine-sensitive phosphorylated peptide at 140,000 M_r was detected in the purified CSCPA fraction (purification in the presence of protease inhibitors, see Chapter 5, section 5:2). These results are characteristic of P-type ATPases, the erythrocyte CSCPA being a member of this class of ATPase. Therefore the maize CSCPA displayed a reaction mechanism similar to the erythrocyte CSCPA. The intensity of phosphorylation at 140,000



KEY:

▬ ▬ ▬ ▬ ; plant peptides

M1 -M10; transmembrane domains

Figure 7.1: Functional domains of the erythrocyte CSCPA (after Carafoli, E., pers. comm.).

M_r was enhanced in the presence of calmodulin and lanthanum (which is an inhibitor of the dephosphorylation process). Phosphorylation at 140,000 M_r was also present in the microsome fraction and showed similar properties to purified extract in being hydroxylamine-sensitive, intensified in the presence of lanthanum and calmodulin and by rapidly turning over. Chapter 5, section 5:3 contains a discussion of the apparent lack of calcium-dependence of phosphorylated intermediate formation in the microsomal fraction; this was the only result which appeared to show significant difference between the putative maize CSCPA and the properties expected for such a system by analogy with mammalian systems, since it would be expected that its activity would be inhibited in the absence of calcium, whether purified or membrane-bound. The enhancement of phosphorylation at 140,000 M_r by calmodulin in the presence of lanthanum in the purified samples provided further supporting evidence that calmodulin interacted directly with the CSCPA; if a calmodulin-dependent phosphokinase were involved, it is unlikely that calmodulin enhancement of phosphorylation would have been observed, since the assay conditions (time period, temperature) were unfavourable for such activity.

After purification in the presence of protease inhibitors, polypeptides of M_r 91,000, 77-69,000, 51,000 and 40,000 were present on neutral SDS-polyacrylamide gels in addition to that at 140,000 M_r. These polypeptides bound calmodulin after electrophoresis (Chapter 4, section 4:2:2), which suggested either that they were proteolysis products of the 140,000 M_r CSCPA which contained the calmodulin-binding domain, or that they were other calmodulin-binding proteins that had co-purified. The first possibility may be considered to be more likely for the majority of these polypeptides. This was deduced from the similarity in size of these polypeptides to major trypsin digestion products of the erythrocyte CSCPA, and from binding of monoclonal antibody EA6 (raised to the maize CSCPA) to polypeptides of 140,000 M_r and 91,000 M_r in the CSCPA purified in the presence of protease inhibitors, and to polypeptides of 69,000, 51,000 and 40,000 M_r in the microsomal fraction prepared in the presence of PMSF (see Chapters 4 and 6

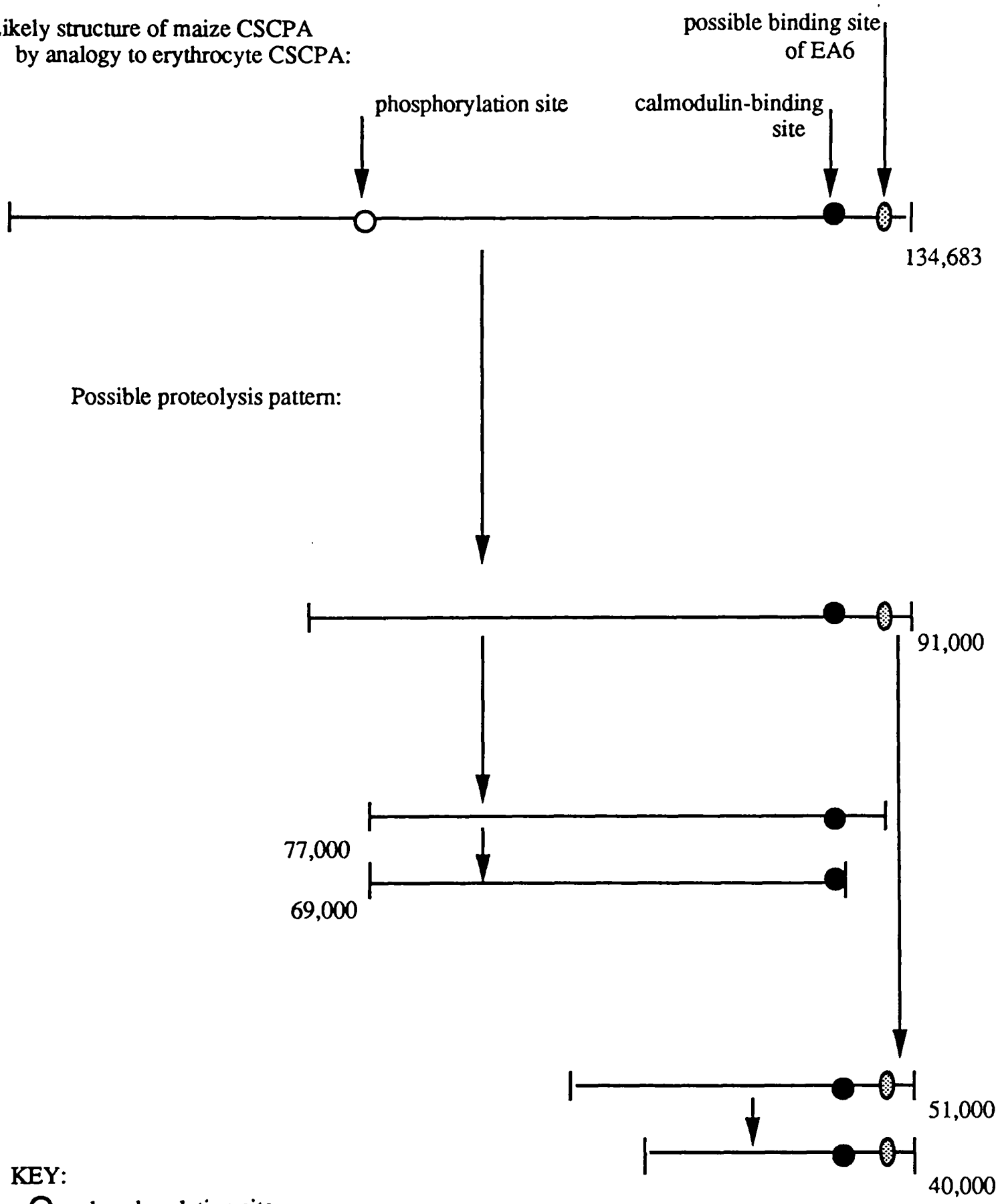


Figure 7.2: Possible proteolysis pathway of maize CSCPA (see text for details).

for details). Assuming that monoclonal antibody EA6 was specific for the CSCPA, this suggests that the polypeptides present on the gels were all proteolysis products of the CSCPA; this could be verified by experiments using the techniques of peptide mapping (Cleveland, 1983).

If the assumption is made that all polypeptides found after purification of the CSCPA in the presence of protease inhibitors are proteolysis products of the maize CSCPA, it is possible from the data presented in this thesis to construct a model for a possible proteolysis pattern. This model takes into account data for calmodulin-binding, phosphorylated intermediate formation and binding of monoclonal antibody EA6. The following additional assumptions have been made:

1) The M_r of the maize CSCPA is the same as that of the erythrocyte CSCPA, as calculated from the cDNA sequence of the latter.

2) The location of the sites of phosphorylated intermediate formation, calmodulin-binding and transmembrane domains are the same as for the erythrocyte CSCPA. These are indicated in Figure 7.1, which shows the location of the major functional domains of the erythrocyte CSCPA. The regions to which the two maize CSCPA peptides show homology are indicated.

3) Monoclonal antibody EA6 is most likely to bind to a hydrophlic region of the CSCPA, rather than a transmembrane domain (see Chapter 6 for details).

Figure 7.2 shows one possible way in which proteolysis of the maize CSCPA may have occurred and indicates a possible location for binding of monoclonal antibody EA6, taking into account these assumptions. Such a scheme requires much further study for it to be validated. This scheme is limited in that it would predict formation of a phosphorylated intermediate by the 91,000 M_r polypeptide (which is not found). Binding of monoclonal antibody EA6 to a 62,000 M_r polypeptide present in microsomes prepared in the absence of protease inhibitors is also not accounted for.

The data presented in this thesis, when considered together, provide the strongest

evidence available indicating that maize cells contain a system similar to the calmodulin-stimulated, calcium pumping ATPase of erythrocytes. Several points arise from this study which merit further consideration and may provide a basis for further research. Firstly, development of a procedure by which maize CSCPA purified in the presence of protease inhibitors could be reconstituted in a functional form into liposomes of known phospholipid content would be advantageous in several ways. If reconstitution could be effected into sealed liposomes then it should be possible to develop an assay to measure calcium transport activity of purified CSCPA. Preliminary attempts to carry out such experiments were made during the course of this study. A cholate dialysis method of reconstitution into asolectin vesicles was carried out as for the ATPase assays (Chapter 4, section 4:2:1) followed by use of a calcium sensitive macroelectrode to measure ATP-dependent calcium loss from the medium and hence calcium uptake into the vesicles (see Appendix I for description of use of the electrode). This method was recommended by Professor E. Carafoli and has apparently been successfully used for the erythrocyte CSCPA (for example, Niggli *et al.* , 1982, 1987). In none of the experiments carried out during this study was it possible to convincingly demonstrate ATP-dependent calcium uptake in reconstituted maize CSCPA samples as compared with controls. Other variations in the protocol described by Niggli *et al.* (1987) were also tested (Niggli *et al.* , 1982; Racker, 1972), again without success. Further studies are therefore required to develop suitable methods. It is possible that the use of Triton X-100 to solubilise membrane proteins from microsomal fractions may have caused problems in the experiments to demonstrate calcium uptake in reconstituted maize CSCPA samples (although Triton X-100 was present in the erythrocyte samples that were reconstituted and apparently showed calcium uptake). Triton X-100 may not have been fully dialysed away during the cholate dialysis procedures used, and its presence may cause the liposomes to be leaky. An alternative approach which could be investigated is the use of a 'Biobeads SM2' absorbent column (as described by Niggli *et al.* , 1987) as an alternative means

Substance affecting activity of erythrocyte CSCPA	Reference
Lysophospholipids	Tokumura *et al.*, 1985
Inositol phosphates	Choquette *et al.*, 1984
cAMP-dependent protein kinase	Neyses *et al.*, 1985
Protein kinase C	Smallwood *et al.*, 1988
Thyroid hormone and retinoic acid	Smith *et al.*, 1989
Calcium-dependent neutral protease (calpain)	Au, 1987; Wang *et al.*, 1988

Table 7.1: Substances affecting activity of erythrocyte CSCPA

of removing Triton X-100. If this proved unsuccessful, then it might be necessary to develop a method of purification using an alternative detergent which was more easily removed. Development of a successful reconstitution method may provide more evidence concerning the functional significance of the maize CSCPA and would provide an assay with which to assess the effects of possible regulators of the CSCPA.

It has been shown in studies of the erythrocyte CSCPA that a variety of substances affect ATPase and calcium transport activity. It was established soon after purification and reconstitution of the CSCPA that acidic phospholipids and long chain fatty acids stimulated activity and substituted for calmodulin (reviewed by Carafoli and Zurini, 1982). It is interesting to note that, in this thesis, it was shown that calmodulin-stimulated ATPase activity was retained after reconstitution into asolectin vesicles; this may be a difference from the erythrocyte CSCPA, but requires further evaluation. Limited proteolysis was also shown to activate the erythrocyte CSCPA, and this provides another possible area of investigation. However, it is not clear whether such phospholipids or proteolysis have any physiological significance in regulating activity of the CSCPA *in vivo*. It is known that a number of other factors which may have physiological significance regulate activity of the CSCPA in red blood cells. These are summarised in Table 7.1. It would be interesting to investigate the effects of inositol phosphates, cAMP-dependent protein kinase and protein kinase C on the maize CSCPA; if any of these were found to have an effect this could provide evidence for physiological significance of likely calcium/inositol phosphate pathway of signal transduction in plant cells (see Chapter 1). Some evidence for such regulation comes from the work of Reddy and Poovaiah (1987) who prepared microsomes from maize coleoptiles using a procedure modified from Dieter and Marmé (1980c) and studied calcium uptake using ^{45}calcium and a filtration system. They showed that addition of inositol trisphosphate (20 μM) to microsomes preloaded with calcium caused an immediate release of calcium. This

suggests that inositol trisphosphate may cause alteration of free calcium levels in the cytoplasm; this study could be extended to investigate the effects of inositol trisphosphate on purified CSCPA.

There is some evidence suggesting that plant growth substances may affect calcium transport and ATPase activity of plant membranes. Kubowicz *et al.* (1982) demonstrated that tissue pretreated with auxin, zeatin or other cytokinins showed altered levels of calcium transport in a partially purified plasma membrane fraction. Concentrations of auxin which promoted hypocotyl elongation were used, and a 100% increase in calcium transport activity was detected. Treatment with zeatin, however, resulted in inhibition of calcium pumping activity; the effect of calmodulin was not investigated. Oláh *et al.* (1983) treated wheat seedlings with the synthetic cytokinin benzylaminopurine and showed increased affinity of a calcium-stimulated ATPase in a microsomal fraction for calcium and calmodulin. The lack of detail in this report concerning the methods used for preparation of the microsomal fraction and assay of calcium-stimulated ATPase make it difficult to assess whether this system is the same as that described in this thesis. The pH optimum of the calcium-stimulated activity was 6.5, which is lower than that described by Robinson *et al.* (1988) and more characteristic of proton-pumping ATPase activity. In addition, the concentration of calcium used was very high (1 mM). However, despite these reservations, investigation of the effects of plant growth substances on the CSCPA described in this thesis could be a fruitful area for study.

It may be considered that further work would be necessary to show directly that the maize CSCPA is located at the plasma membrane. This could be approached by means of preparing a purified plasma membrane fraction and establishing its identity rigorously (see Chapter 1 for details) before solubilising plasma membrane proteins and applying to a calmodulin-affinity chromatography column. The high degree of sensitivity to proteolysis observed for the maize CSCPA and also reported for the erythrocyte CSCPA (Carafoli and Zurini, 1982) means that it is desirable to carry out purification

as rapidly as possible, a constraint which must be borne in mind if experiments to prepare purified plasma membrane were carried out. Alternatively, it may be possible to raise other monoclonal antibodies of class IgG to the intact maize CSCPA and to use these in immunoelectron microscopy. Such an approach would require generation of monoclonal antibodies which recognised regions of the CSCPA found only in this type of ATPase, since a non-calmodulin stimulated ATPase is probably present at the endoplasmic reticulum of plant cells (Buckhout, 1983, 1984).

There has been debate concerning whether calmodulin-stimulation is a feature of all ATP-dependent calcium transporting systems located (or probably located) at the plasma membrane (see Chapter 1 for details and references). It remains a possibility that other mechanisms mediating calcium efflux from plant cells are present at the plasma membrane. It is known that a few cell surface calcium transport ATPases in mammalian cells are not stimulated by calmodulin. An example of such a calcium transporting system is that found on the plasma membrane of corpus luteum (Verma and Penniston, 1981). It is possible that some plant cells may be similar to animal cells in this respect, having more than one type of calcium pumping ATPase. Further studies are required to investigate these possibilities; it may be that calmodulin-stimulation is a feature of some cell types (e.g. maize coleoptiles) and not others (e.g. pea roots, Butcher and Evans, 1987a and b). One relatively easy way by which this could be investigated would be to prepare microsomal or plasma membrane enriched fractions from a variety of plant species, and to assay for formation of a phosphorylated intermediate at or near 140,000 M_r which was stimulated by lanthanum and calmodulin.

It is to be hoped that further studies concerning the system described in this thesis will be carried out as a means towards furthering understanding of regulation of $_p[Ca]_{cyt.}$ in higher plants. Such studies, when combined with more information on other calcium transporting systems may lead eventually to a description of the way in which extracellular signals may be transduced by intracellular signals, including calcium ions,

into the appropriate biochemical or developmental response.

Appendix I

Calcium uptake by microsomal vesicles

I.1 METHODS

Microsomal vesicles from *Zea mays* L. coleoptiles were prepared in the presence of PMSF as described (Chapter 2, section 2:2:2); the pellet from the second centrifugation at 48,000xg was resuspended (at 4 mg protein/ml) in Buffer B1 (i.e. Triton X-100 was not present). All buffers throughout extraction and assay were made up with ultra-pure water in EGTA-washed plastic vessels to minimise calcium levels. Microsomal fractions were handled using pipette tips which had been cut to expand the tip diameter to minimise damage during preparation and assay.

A calcium-sensitive electrode (Orion Research Inc., Mass., product number 9320BN) was set up according to the manufacturer's instructions. The sensing module contained a gelled organophilic membrane with a calcium selective ionophore based on ETH 1001 (Simon and Carafoli, 1979). Basic principles of calcium-sensitive electrode theory and operation may be found in Affolter and Sigel (1979) and Sigel and Affolter (1987). The reference electrode used was of the double-junction type (Orion Research Inc., Mass.,

product number 900200); the inner and outer chambers were filled with appropriate solutions as specified by Orion (product numbers 90-00-02 and 90-00-11 respectively). Electrodes were set up freshly each day, and pre-equilibrated in $1x10^{-4}$ M calcium solution (BDH calcium standard stock solution, 10 mmol/litre, diluted with ultra-pure water) for at least one hour. Electrodes were connected to an Orion model SA 720 pH/ISE meter, and an electronic chart recorder (Rikadenki, Kogyo, Japan, model R-01).

The electrode was calibrated by addition of known amounts of calcium to ultra-pure water, and by addition of known amounts of calcium to assay medium. The curves obtained were overlayed such that the amount of free calcium in incubation medium could be determined. Over the mV range of the assays, the change in calcium concentration per mV was estimated and used in calculation of rates of calcium uptake by vesicles.

Assay components were made up as follows:-

1) Incubation medium; Basic Buffer (Chapter 2, section 2:2:2) with 0.5% (w/v) BSA, 0.1 M KCl, 1 mM $MgCl_2$, 1 mM DDT, 1 x 10^{-6} M oligomycin. Maintained at 25°C in a water bath.

2) ATP regenerating system; as used by Ashley and Moisescu (1977), the reaction being as below:

creatine phosphate + ADP $\longrightarrow$ creatine + ATP

ATP (10 mM) and creatine phosphate (100 mM) were mixed and made up in incubation medium, a separate stock of creatine kinase (200 units/ml) also being prepared. These were kept on ice and appropriate volumes of each added to the assays, giving final concentrations of ATP (1 mM), creatine phosphate (10 mM), creatine kinase (30 units).

3) Calmodulin; stored and prepared as described in Chapter 2, section 2:2:4. Final concentration in assay 0-1.0 μM.

Assays were carried out in a perspe incubation vessel which held both electrodes and permitted assays to be carried out in a small volume (1.5 ml). A narrow injection port was also present. Assays were stirred at a constant rate with a non-vortexing magnetic stirrer (magnetic follower ring, type LL, 12 mm diameter, Gallenkamp, U.K.) driven by a 'Turbo jet' magnetic stirrer (Cam Lab, Cambridge, U.K.).

Assays were carried out in the following steps:

1) Incubation medium was placed in the incubation vessel, and the mV reading allowed to stabilise.

2) ATP regenerating system was added, the reading allowed to stabilise and calmodulin added if required.

3) Small volumes (2 μl) of 1 x 10^{-2} M calcium standard solution were added as required to bring the mV reading back to that of incubation medium alone.

4) An aliquot of microsomal vesicles (150 μl, protein content 600 μg) was added and changes in mV reading followed on a chart recorder over 10-15 minutes. Addition of calcium ionophore A23187 (Sigma, magnesium salt) was made as required (5 μM final concentration, from stock in ethanol). Controls were carried out with addition of ethanol alone; A23187 added to incubation medium only did not affect electrode reading.

I.2 RESULTS

Calculations of rates of calcium loss from the assay medium (equivalent to uptake by vesicles after controls taken into account) were made from linear parts of traces. It was not possible to calculate total amounts of calcium taken up because of non-linear changes in mV reading which took place over the first 2-3 minutes after addition of microsome sample. This was most likely to be a mixing phenomenon; variation in its pattern (compare traces for +ATP in Figures I.1 and I.2, for example) between assays was evident, hence total uptakes could not be calculated. This was not improved by

initiating the assay by addition of ATP; if this was carried out, apparent 'uptake' occured in the presence or absence of microsomal vesicles, as a consequence of complexing of calcium by ATP.

i). Demonstration of ATP-dependence of calcium uptake.

Figure I.1 shows that in absence of ATP, the rate of uptake was zero. No uptake occured when boiled microsomes were incorporated into the assay. In the presence of ATP, the rate of calcium uptake in this representative experiment was 0.26 nmol min^{-1} $(mg\ protein)^{-1}$. Due to the nature of these experiments, it was not possible to repeat assays within one experiment (each assay taking 20 minutes, and the microsomal fraction remaining active over about 2 hours). The data presented in Table I.1 shows rates of ATP-dependent calcium uptake in 15 different experiments. Uptake in the absence of ATP was zero in every case, in the presence of 1 mM ATP the average rate of calcium uptake was 0.28 nmoles min^{-1} $(mg\ protein)^{-1}$.

ii). Effect of calmodulin on rate of ATP-dependent calcium uptake.

Calmodulin stimulated the rate of calcium uptake in microsomal fractions, as shown in Figure I.2. In this representive experiment, uptake in the presence of 1 mM ATP was 0.37 nmoles min^{-1} $(mg\ protein)^{-1}$, and with ATP and 1 μM calmodulin was 0.7 nmol min^{-1} $(mg\ protein)^{-1}$, the calmodulin-stimulated component being 0.33 nmol min^{-1} $(mg\ protein)^{-1}$. The effect of varying calmodulin concentrations is shown in Figure I.3; calmodulin stimulation was apparent at low concentrations (0.005 μM) and was maximal at 0.3 μM calmodulin.

iii). Effect of ionophore A23187 on calmodulin-stimulated calcium uptake.

Addition of ionophore A23187 (5 μM final concentration) caused a rapid increase in calcium concentration of the medium, indicating loss of calcium from the vesicles (Figure I.2). Addition of an equal volume of ethanol alone did not cause an increase in medium calcium concentrations (Figure I.2).

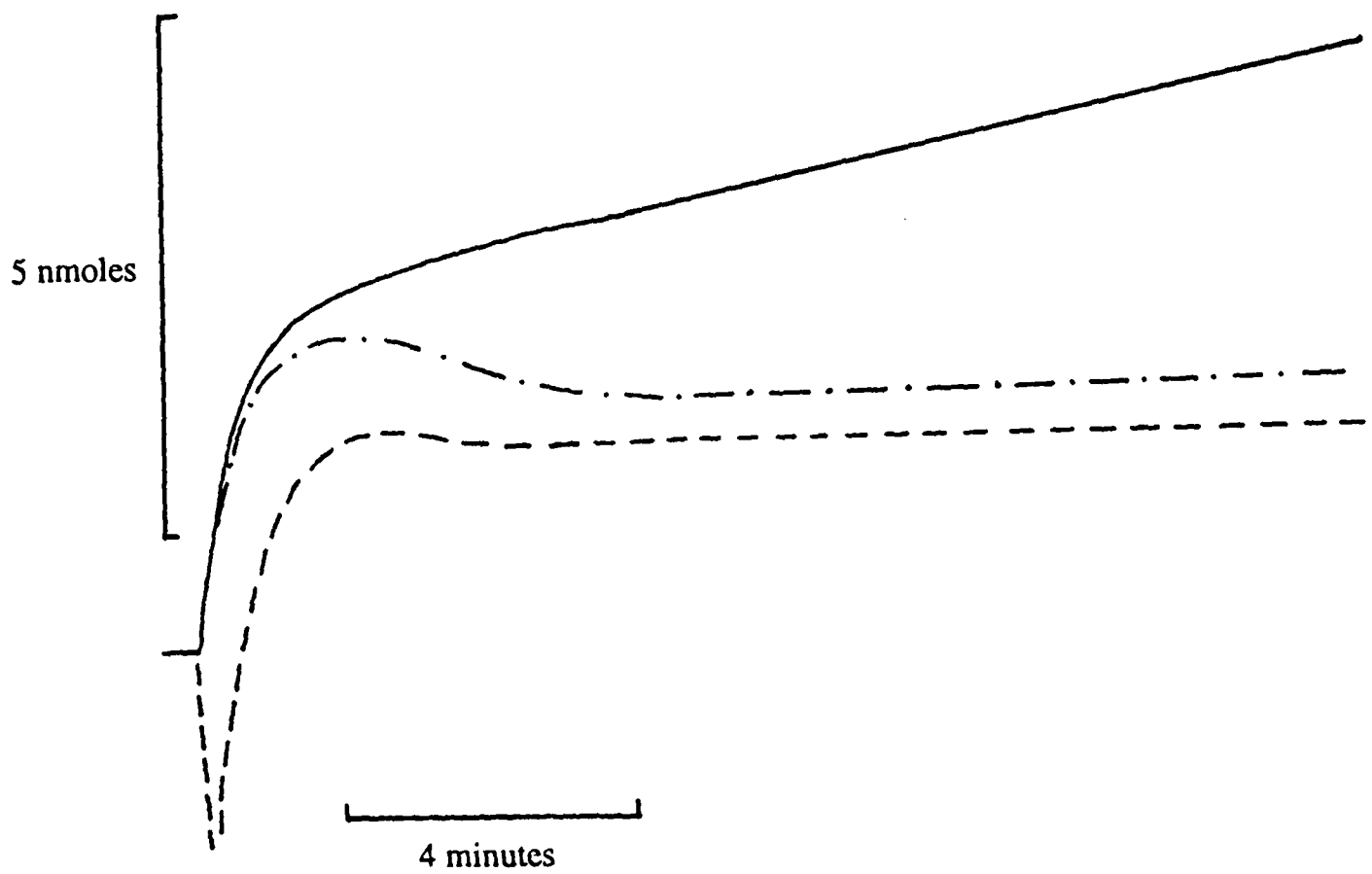
Figure I.1: ATP-dependent calcium uptake in microsomes.

— — — Boiled microsomes.

— - — Fresh microsomes without ATP.

——— Fresh microsomes with 1 mM ATP.

Traces are re-drawn from one representative experiment out of fifteen.


5 nmoles
4 minutes

Rate of calcium uptake (nmol min^{-1} $(mg\ protein)^{-1}$)		
Boiled microsomes	-ATP	+ATP
0	0	0.23
0	0	0.29
--	0	0.35
--	0	0.39
--	0	0.29
--	0	0.19
--	0	0.18
--	0	0.26
--	0	0.21
--	0	0.41
--	0	0.19
--	0	0.37
--	0	0.21
--	0	0.35
--	0	0.35
		$\bar{x} = 0.28$
		$\sigma = 0.08$

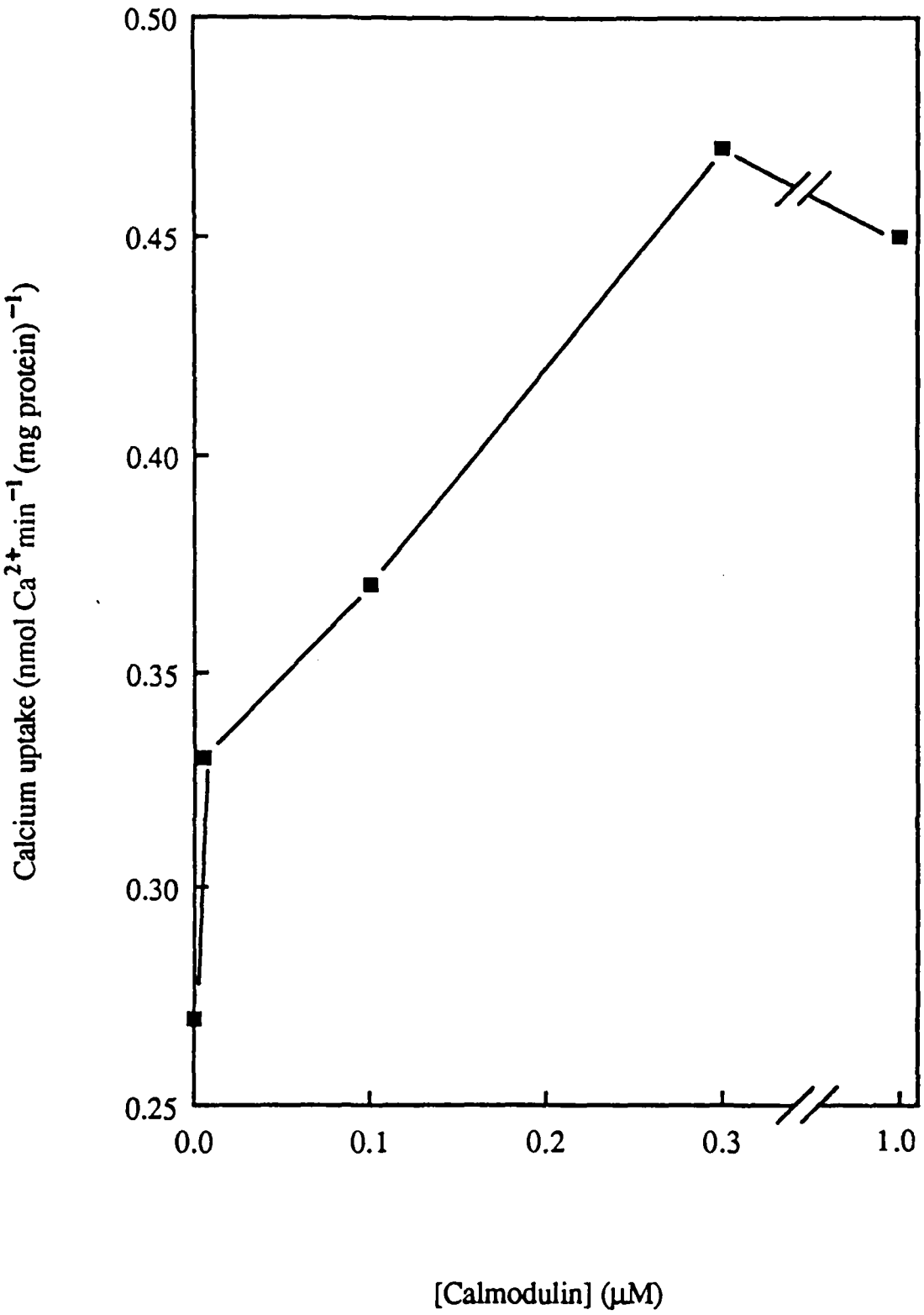
Table I.1: Collation of data for uptake of calcium into microsomes, measured using a calcium-sensitive electrode.

$\bar{x}$ = mean

σ = standard deviation

Figure I.3: Dose-response curve for effect of calmodulin on ATP-dependent calcium uptake in microsomes.

Representative of two experiments.


0.50
0.45
0.40
0.35
0.30
0.25
0.0
0.1
0.2
0.3
1.0
Calcium uptake (nmol Ca^{2+} min^{-1} (mg protein)$^{-1}$)
[Calmodulin] (μM)

Appendix II

Amino-acid sequencing of fragments of maize CSCPA

II.1 METHODS

Samples of maize CSCPA were purified in the absence of PMSF, as described in Chapter 2, section 2:2:3 with asolectin added to 100KS and to Buffer C. After application of sample to the affinity column, the column was washed for 18 hours with Buffer C containing asolectin. Prior to elution, the buffer was changed to Buffer C containing 0.025% (w/v) phosphatidylcholine (from egg yolk, Lipid Products, South Nutfield, Surrey, U.K.) for 30 minutes. Calmodulin-binding proteins were eluted with Buffer D containing 0.025% (w/v) phosphatidylcholine. Thimerosal (10 mM final concentration, Sigma, sodium salt) was added to the peak fractions, which were stored at -70°C. Samples were delivered to Zürich, and were sequenced by P. James (Laboratorium für Organische Chemie, Eidgenössiche Technische Hochschule) after cyanogen bromide digestion and separation on a high performance liquid chromatography column.

II.2 RESULTS

Two short peptides (CNBr1 and CNBr2) were sequenced:

Peptide CN Br1	GIAGSDAAR
For comparison:	
sodium/potassium-transporting ATPase,	GIAGSDVSK
α-chain precursor (percent	
similarity 77%)	
calmodulin-dependent erythrocyte	MGIAGTDVAK
Ca^{2+}-pumping ATPase	
(percent similarity 80.0%)	
Peptide CN Br2	THPEFLLDQD
For comparison:	
calmodulin-dependent erythrocyte	THPEFRIEDS
Ca^{2+}-pumping ATPase	
(percent similarity 80.0%)	

Appendix III

Production of monoclonal antibodies to the maize CSCPA

III.1 INTRODUCTION

This Appendix contains data concerning the initial selection and characterisation of the monoclonal antibodies raised to the maize calmodulin-stimulated calcium pumping ATPase (CSCPA) used in the studies described in Chapter 6.

III.2 METHODS

The following work was carried out under the supervision of Dr. F.M. Dewey, who performed all the work with animals within the guidelines stipulated by the Home Office. Her personal animal licence number is PIL 30/00267.

III.2.1 Production of monoclonal antibodies

i) Preparation of antigen

Samples of the calmodulin-stimulated, calcium pumping ATPase (CSCPA) were purified from maize coleoptiles by means of calmodulin-affinity chromatography (column

buffers without PMSF or asolectin, and a two hour column wash). The samples were prepared for injection by dialysis overnight against three changes of 500 volumes of phosphate buffered saline (PBS, 0.2 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4 in deionised water). The dialysed CSCPA is referred to below as the 'antigen'.

ii) Immunisation of mice and collection of polyclonal serum.

Three female mice, haplotype BALB/c, were injected twice with 300 μl of the dialysed antigen (protein content 6-60 μg, by modified Lowry assay) at two week intervals. A booster injection was given 4 days before the fusion was carried out. All injections were made without adjuvant. Mice were bled from a tail vein 4 or 5 days after the second and booster injections. The blood was allowed to clot overnight at 4°C and erythrocytes subsequently pelleted at 250xg for 1 minute (Dupont Sorvall 'Microspin 12S'). Antiserum was diluted 10-fold with PBS and sodium azide added to 0.02% (w/v) from a stock of 2% (w/v) in distilled water. The antiserum was stored at 4°C until required.

iii) Growth and maintenance of cell lines.

Cells of a myeloma cell line SP2/0-Ag-14, were cultured in tissue culture medium (TCM) (RPMI 1640 with 2 mM glutamine (Flow Labs), penicillin 560 units/ml, and streptomycin 112 μg/ml, supplemented with 10% (v/v) foetal bovine serum (FBS, Flow Labs)) in a humid atmosphere containing 5% (v/v) CO_2 at 37°C. The foetal bovine serum had been screened for support of myeloma and hybridoma cell growth (Harlow and Lane, 1988). If required, gentamycin (0.2 mg/ml) and/or kanamycin (50 μg/ml) were added to the TCM. All cell culture work was carried out using sterile techniques in a laminar flow hood. The cells were maintained at a density of 10^6 to 10^7 cells/ml by transferring 0.5-1.0 ml of the cell suspension to 9 ml fresh medium every 3 to 5 days.

iv) Fusion of spleen and myeloma cells.

Four days after the booster injection, the spleen was removed from the mouse chosen and placed in a 'Universal' tube (Sterilin, UK) containing TCM with 20% (v/v) FBS.

Using sterile hypodermic neddles, the spleen was transferred to a large Petri dish and teased out to release splenocytes.

Fusion of spleen cells with myeloma cells was carried out as described by Kennet *et al.* (1978), which is based on the method of Köhler and Milstein (1975). The spleen cell suspension (approximately 10^7 cells) was transferred to a 50 ml sterile Falcon tube, and allowed to stand for about three minutes to allow any large clumps to settle, after which the supernatant was decanted. Approximately 10^7 SP2 cells in exponential growth phase were transferred to a second Falcon tube. Both tubes of cells were centrifuged separately at 250xg for 5 minutes, and the pellets resuspended and washed in TCM without FBS and recentrifuged. The cells were gently resuspended in 5 ml RPMI without FBS, pooled and centrifuged again. The pellet was carefully resuspended in 30% (v/v) polyethylene glycol (PEG, in serum free TCM) for a total of 7 minutes including a 2 minute centrifugation step at 500xg. PEG was removed carefully and cells slowly resuspended in 5 ml non-supplemented TCM. Finally the cells were further diluted into 45 ml TCM with 20% (v/v) FBS and plated out into 10, 96-well plates at 50 μl per well.

v) Selection and maintenance of antibody-secreting hybridoma cell lines.

Twenty-four hours after the myeloma and spleen cells were fused, 50 μl of double strength HAZA selective medium (as described by Foung *et al.* , 1982; 2 μg/ml azaserine and 200 μM hypoxanthine in TCM) were added to each well. Wells were fed at 3 or 4 day intervals by adding 50 μl of normal concentration selective medium; after 7 to 10 days wells containing hybridomas were identified and supernatants screened by ELISA for antibodies. Positive clones were fed after 10 days with 50 μl of non-selective medium and when cells became confluent, they were successively transferred to 24- and then 6-well plates. Positive cell lines were bulked up into petri dishes and then cell culture flasks. As the cells were transferred to larger culture vessels, the amount of FBS was reduced in steps of 5% until the cells were growing in TCM supplemented with 5% (v/v)

FBS. Cell free hybridoma supernatants were stored at 4°C with 0.02% (w/v) sodium azide. Positive cell lines were cloned by limiting dilution once or twice until all the clones recognised the antigen to the same extent as the parent cell lines.

Cell lines of interest were stored frozen in aliquots under liquid nitrogen. Care was taken to only freeze actively growing cells. Cell suspensions (approximately 50 ml) were centrifuged in sterile Falcon tubes at 250xg and the pellet gently resuspended in 600 μl of freezing mixture (8% (v/v) dimethyl sulphoxide (DMSO) and 92% (v/v) FBS). Subsequently, 200 μl aliquots of this mixture were transferred to freezing vials, which were firmly sealed. These vials were placed in cotton wool within a polystyrene container and stored in a -70°C freezer for 16 hours to allow the cells to cool gradually. After this period, the vials were placed in supporting canes and quickly transferred to a Dewar containing liquid nitrogen. When these cell lines were required for use, they were removed from the Dewar, thawed rapidly by warming in the hand, and transferred to 5 ml of warm TCM with 20% (v/v) FBS in a small petri dish. The cells were immediately replaced in the incubator, and were bulked up into large petri dishes followed by cell culture flasks as appropriate.

III.2.2 Assay of antibodies by ELISA

An antibody capture method was used, based on that of Dewey and Brasier (1988). Disposable polystyrene microtitre wells in 12-well strips (Flow Labs, 'Titertek' 1x12 well (FB) 'Microstrip') were used; all test volumes were 50 μl per well. Wells were coated overnight at 4°C with antigen that had been dialysed overnight into PBS with 0.02% (w/v) sodium azide (PSBA). Early in the development of the assay, using polyclonal antiserum, the amount of antigen was varied in steps of doubling dilutions from undiluted (straight out of the dialysis bag) to 128-fold diluted into PBSA with 0.05% (v/v) Tween-20 (PBSAT). Subsequently, the dialysed antigen was diluted routinely 10-fold into PBSAT before being dispensed into ELISA wells. After incubation, unbound

material was removed by inverting and banging the plates, followed by washing four times with PBSAT and once with PBSA. The wells were rinsed briefly with distilled water and air dried in a laminar flow hood. Plates were stored in sealed polythene bags at 4°C for a maximum of eight months.

Subsequent steps in the ELISA were carried out at room temperature. Between each addition, plates were emptied by banging and washed four times for five minutes with PBSAT and dried onto absorbent paper. The following primary antibodies were assayed:

1) Cell free hybridoma supernatants containing monoclonal antibodies:

2) Polyclonal antiserum from the mouse used to produce monoclonal antibodies:

3) Affinity purified monospecific polyclonal antibody 97.1 raised in rabbits against the intact erythrocyte calmodulin-stimulated calcium pumping ATPase, supplied from F. Kessler, Eidgenössiche Technische Höchschule, Zürich.

Dilutions were made into PBSAT, and incubation with primary antibody was for 3 hours. The secondary antibody was Sigma goat antimouse polyvalent immunoglobulin horseradish peroxidase conjugate (catalog number 0412) diluted 200-fold into PBSAT; incubation was for 1 hour. Substrate was freshly prepared from a stock of 2.5 mg/ml tetramethylbenzidine in dimethyl sulphoxide diluted 10-fold into acetate buffer (0.2 M sodium acetate, 0.0025% (v/v) H_2O_2, 2 mM citric acid). Plates were incubated with substrate for 30 minutes, and the reaction terminated by addition of 2.5 M H_2SO_4. The optical density of the wells was read immediately on a Titertek 'Miniscan' or Dynatech MR 700 Microplate reader at 450 nm, blanked against an empty well. In each experiment controls with TCM were included, and checks were made for non-specific binding of the various antibodies.

III.2.3 Estimation of the amount of protein bound to ELISA wells

A very sensitive protein assay was carried out using Janssen 'Aurodye forte' and a Millipore membrane as described by MacDonald *et al.* (1989). Pieces of polyvinylidene difluoride (PVDF, Millipore) membrane were wetted with 100% methanol for 2 minutes, and rinsed in distilled water for 1 minute. The membranes were air dried, placed on a piece of damp filter paper in a plastic petri dish and samples of the protein to be assayed were dotted onto the strips in 3 μl aliquots. A standard series of dots was made from a solution of bovine serum albumin (BSA) in PBS, in doubling dilutions from a maximum of 0.1 mg/ml. Samples were taken from the wells of the ELISA plate that contained purified CSCPA in doubling dilutions, both immediately after the solutions were placed in the wells, and after the wells had been incubated overnight. The protein samples were allowed to dry onto the membranes, which were rewetted with methanol and water as above. Excess water was shaken off, and the membranes immersed in an excess of 'Aurodye Forte' (Janssen, from ICN Biomedicals, High Wycombe, Bucks., U.K.). The dishes were placed on a rocking table at a room temperature and incubated for 30 minutes. The membranes were then washed in several changes of excess water, and dried between filter papers. Estimation of the amount of protein bound to the walls of ELISA plates was made by visual comparison between the standard strips with known protein content and the test strips. The estimation of the protein content of the solutions in the ELISA wells after overnight incubation was subtracted from that before incubation to produce an estimation of the amount of protein bound to the PVDF membrane.

III.2.4 Cloning cell lines by limiting dilution

To ensure that cell lines of interest were monoclonal, they were cloned twice by limiting dilution. This was carried out by diluting cells in TCM with 20% (v/v) FBS (see Harlow and Lane, 1989). Plates were examined for clones after 7 to 10 days and fed

when necessary. Dilution plates with less than one-third of the wells containing cells were examined for the presence of distinct single colonies. Supernatants from single clones were tested by ELISA when the colony of cells covered at least one-third of the well. From the results of this assay, cell lines were either selected and grown in bulk or recloned until supernatants from all the clones tested positively, giving approximately the same absorbance readings by ELISA.

III.2.5 Periodate treatment of antigen-coated plates

This was based on the method of Wycoff *et al.* (1987). Antigen coated plates were first blocked with 0.5% (w/v) BSA (immunoglobulin-free) in PBS (blocking buffer) for 20 minutes, and then washed three times with sodium acetate buffer (50 mM, pH 4.5). Wells were filled with 100μl acetate buffer with or without 20 mM sodium metaperiodate, and incubated overnight at 4^{o}C. Plates were washed four times for five minutes each with blocking buffer, dried and the normal ELISA protocol followed.

III.2.6 Determination of immunoglobulin class and sub-class

The monoclonal antibodies in the hybridoma supernatants were assigned to the respective class and sub-class using a mouse monoclonal typing kit (Serotec, Kidlington, Oxford, U.K.), which utilised the principal of double immunodiffusion as described by Ouchterlony (1967). The agar plates supplied had wells punched out such that there was one central well surrounded at equal distances by six other wells. An aliquot (75 μl) of the hybridoma supernatant to be tested was pipetted into the central well. Into the six outer wells, 10 μl of each of the six antisera directed against the classes and sub-classes IgG1, IgG2a, IgG3, IgM and IgA were pipetted. Immunoprecipitate lines were visible after 48 hours, which permitted identification of the antibody class by comparison with immunoprecipitate lines formed in the controls of known class supplied with the kit.

III.3 RESULTS

III.3.1 Estimation of amount of protein bound to wells of ELISA plates

Microtitre wells of ELISA plates were coated with dialysed CSCPA (purified without PMSF or asolectin and with a two hour column wash; referred to as the 'antigen'). 'Aurodye' estimate of the amount of protein bound to the wells after overnight incubation (see Figure III.1) indicated that before incubation there was 2.25 ng of protein in wells containing 50 μl of CSCPA (diluted 18-fold) and 0.55 ng after incubation. This gave an estimate of 1.7 ng of protein bound to the well.

The 'Aurodye' protein assay method indicated that in the total (approximately 4 ml) dialysed CSCPA fraction there was a total of 1.4 μg of protein (as derived from an estimation of 2.25 ng protein in 50 μl of 8-fold diluted CSCPA). This estimate was low compared with that of a modified Lowry assay (Bensadoun and Weinstein, 1976) which gave a value of 0.1 mg protein in the total dialysed CSCPA fraction. Estimation of protein content in this fraction by the method of Warburg and Christian (1941) gave the highest value of 1.0 mg protein. These discrepancies in estimation of protein content indicate the problems involved when assaying samples of low protein content. Use of the 'Aurodye' method was made for determination of the amount of protein bound to the ELISA plate wells because it was the only method which was sufficiently sensitive to allow measurement of protein concentration in very small samples (3 μl) containing low amounts of protein.

III.3.2 Indirect evidence for nature of antigen bound to ELISA wells

It was of interest to determine whether CSCPA bound to ELISA wells was in its intact form or proteolysed. After purification in the absence of PMSF (but with asolectin in the column buffers) SDS polyacrylamide gel electrophoresis of the EGTA-eluted peak fraction containing the CSCPA showed three polypeptides of 62,000 66,000 and 72,000

M_r when stained with Coomassie Blue (Chapter 4, Figure 4.11). The same fraction also showed calmodulin-stimulated ATPase activity (Chapter 4, Figure 4.10) and hence contained the CSCPA. It was not known whether degradation of the CSCPA occured during electrophoresis and whether or not the sample used to coat the ELISA wells contained intact CSCPA. It was shown (Chapter 4, Figure 4.13) that the anti-erythrocyte CSCPA polyclonal antibody 97.1 recognised only intact CSCPA at 140,000 M_r after immunoblotting. Wells of ELISA plates coated with approximately 2 ng of dialysed CSCPA (as estimated by the 'Aurodye' assay) were incubated with doubling dilutions (from 100-fold dilution into PBST) of antibody 97.1. After ELISA the A_{450} values for the wells incubated with dilutions of 97.1 were the same as for wells incubated with PBSAT alone. This indicated that the intact CSCPA was not bound to the wells or that it bound in such a way that the amino acid residues recognised by antibody 97.1 were not available. Therefore it was concluded that the ELISA wells were probably coated with proteolysis products of the CSCPA and that the mice were likely to have been immunised with these proteolysis products.

III.3.3 Assay of polyclonal antiserum by ELISA

Before proceding with fusion of splenocytes and myeloma cells it was essential to ensure that the mice were producing antibodies to the antigen after immunisation. Such assays also permitted development of a suitable rapid screening method which was used in early evaluation of the antibodies secreted by the hybridoma cells. Antiserum was collected from the three mice (coded by ear punching and designated as NE, LE and RE). For the initial assays of antiserum (taken after two immunisations) a chequer-board ELISA with antiserum diluted by doubling dilutions across the columns of the plate and antigen diluted in doubling dilutions across the rows of the plate was set up. After ELISA the A_{450} of the wells was determined. The end point was not reached at the highest dilutions, 128-fold dilution of antigen (about 0.16 ng of antigen bound to the well) and

800-fold dilution of antiserum. The assay was repeated using ELISA plates coated with approximately 1 ng of antigen and antiserum diluted in nine doubling steps from 800-fold to 409,600-fold. The results obtained are shown in Figure III.2. From this it can be seen that the titre for each of the three mice was high; mice RE and NE showed the highest titres, with absorbance readings twice that of background at 512,000-fold dilution and 256,000-fold dilution respectively. These high titre values indicated that the mice were responding well to the antigen after two immunisations, and hence were given a booster injection of antigen in preparation for the fusion. A similar assay was performed with antiserum taken from the mice three days after the booster injection (data not shown) and mouse NE was chosen for fusion of its splenocyte cells with myeloma cells.

III.3.4 Assay of antibodies secreted by hybridomas by ELISA

Splenocyte and myeloma cells were fused as described. Cells from the fusion process were plated out into 10, 96-well cell culture plates at 50 μl per well. The total number of wells which contained hybridomas was 29, and of these six wells contained cell lines which secreted antibody to the antigen bound to ELISA wells. These six cell lines were selected on the basis of producing absorbance readings four times that of the control (TCM with 20% FBS), with the exception of cell line JF9 which had an absorbance about twice that of control. The six positive hybridoma cell lines from this first screening process were selected for further culture; ELISAs were performed at suitable stages during this process to verify that the hybridomas continued to secrete antibody. It was found that two of these cell lines (JF9 and DF5) were apparently unstable, as they no longer secreted antibodies that recognised CSCPA by ELISA after transfer to 24-well plates. The antibodies from the six cell lines were assigned to their respective class by means of an Ouchterlony-type assay. From this assay it was found that cell line JF9 was no longer secreting antibodies and cell line DF5 was secreting non-specific antibodies of class IgM, but they did not recognise the CSCPA. These two cell lines were not cloned

by limiting dilution but were maintained to provide additional controls in some of the assays described in Chapter 6, section 6:2.

During the course of maintaining the cell lines of interest, control ELISAs were carried out to check that non-specific binding effects were not interfering with the results obtained. Such controls included the following: uncoated plates; assays without primary antibody, secondary antibody or substrate; assays of RPMI alone and assays of other monoclonal antibody-containing supernatants produced by Dr. F. M. Dewey to fungal surface antigens unrelated to the CSCPA. In all cases the A_{450} values were of similar magnitude to those of TCM alone. ELISAs were also carried out with supernatants from cell lines that had been frozen and thawed to check that they were still capable of producing antibodies. Hybridoma supernatants stored at 4°C were periodically checked to ensure that the antibodies were active after storage at this temperature.

III.3.5 Cloning of cell lines by limiting dilution

Cell lines EA6, CC10, FF7 and ID1 were cloned by limiting dilution after reaching a stage where cells were actively growing in wells of 24-well cell culture plates. The original culture was diluted in the following steps: 100-fold, 10,000-fold, 50,000-fold and 100,000-fold. These dilutions were each aliquoted (in 100 μl samples) over 48 wells of a 96-well cell culture plate and growth of the colonies was monitored over three days. The results from the first cloning by limiting dilution are shown in Figure III.3, in which the data has been expressed by taking the absorbance reading of the polyclonal antiserum as 100% . From this data it can be seen that supernatants from all the colonies at the 10,000-fold dilution of cell lines ID1 and FF7 were similar (in the region of 50% absorbance for ID1 and 100% absorbance for FF7). Since the initial plating of the fusion products was in effect a dilution step which increased the chances of cell lines being derived from a single fusion product, cell lines ID1 and FF7 were regarded as being monoclonal at this stage and colonies from the 10,000-fold dilution were selected

for further culture and the supernatants used in subsequent assays. Colonies from a 10,000-fold dilution of cell line CC10 all showed a similar, but low, level of binding (about 12% absorbance). This cell line was probably monoclonal at this stage, but to prove that this was so, was cloned a second time by limiting dilution. It appeared that cell line EA6 was not monoclonal at this stage, since colonies from 10,000-fold dilution showed two levels of reaction on the ELISA plates, some colonies giving high absorbance values (around 100%) and others low absorbance values (around 30% , only slightly higher than the TCM background value of 22% in this experiment). One of the colonies with high absorbance values was selected for further culture and when actively growing was cloned a second time by limiting dilution.

The results from the second cloning procedure are shown in Figure III.4. From this it became clear that cell line CC10 was monoclonal. Cell line EA6 continued to behave erratically, some daughter cell lines apparently not secreting antibodies. It was considered likely that this cell line had an unstable assortment of chromosomes. However, preliminary secondary screens (data not shown) indicated that this cell line was of interest, and hence colonies with high levels of cross reaction from the second cloning stage were selected for further culture and used in subsequent experiments.

III.3.6 Determination of immunoglobulin class

The results obtained by double immunodiffusion (data not shown) indicated that all the monoclonal cell lines (EA6, CC10, ID1 and FF7) were of IgM class.

III.3.7 Periodate treatment of ELISA plates

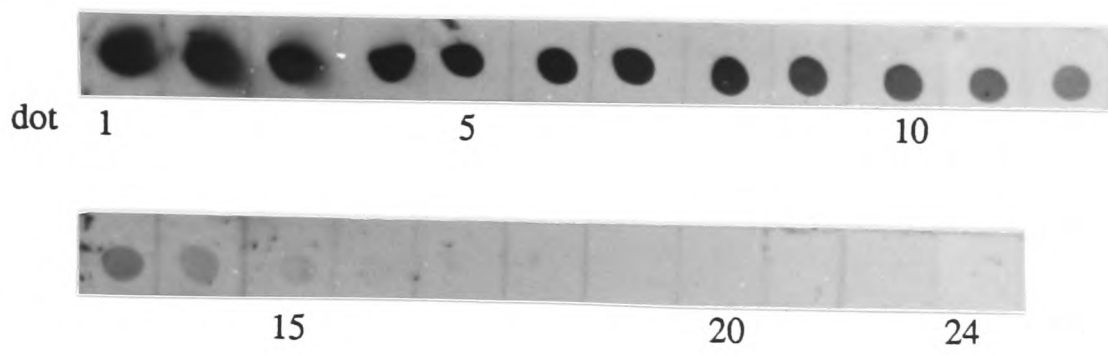
The results from periodate treatment of the CSCPA preparation bound to ELISA wells are shown in Figure III.5. From this it can be seen that monoclonal antibodies EA6 and CC10 apparently recognised peptide epitopes, since the extent of reaction with the

antigen was the same whether or not the wells had been treated with periodate. Monoclonal antibodies FF7 and ID1 gave lower absorbance values after the antigen had been treated with periodate, indicating they recognised carbohydrate epitopes. Polyclonal antiserum also contained a proportion of antibodies which recognised carbohydrate epitopes. The control antibodies DF5 and JF9, together with TCM gave low absorbance readings in the presence or absence of periodate.

Figure III.1: Aurodye estimation of amount of protein bound to wells of ELISA plates.

See text for details.

a BSA standard dots



b Samples from ELISA

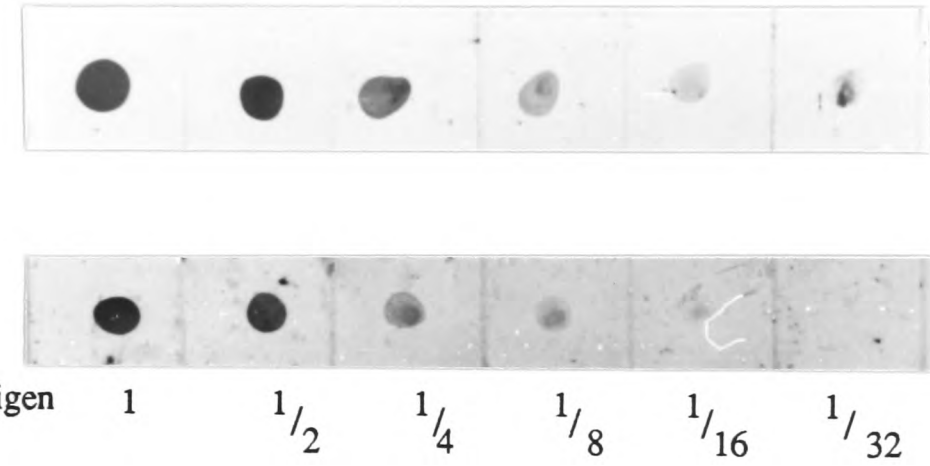
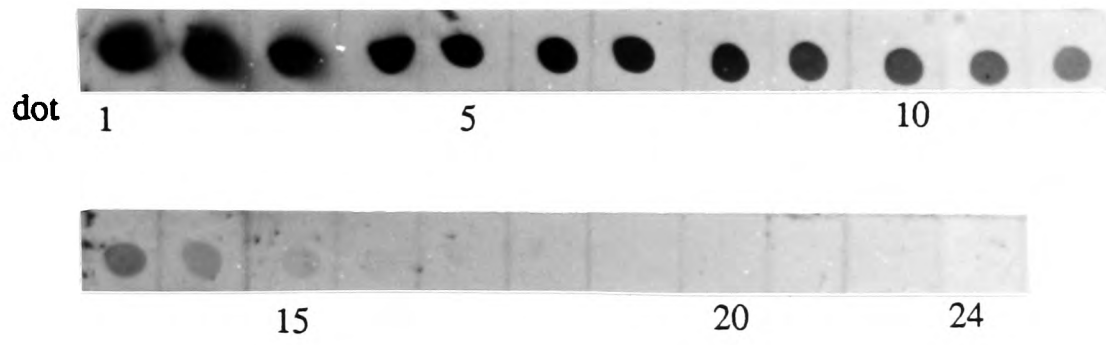


Figure III.1: Aurodye estimation of amount of protein bound to wells of ELISA plates.

See text for details.

a BSA standard dots



b Samples from ELISA

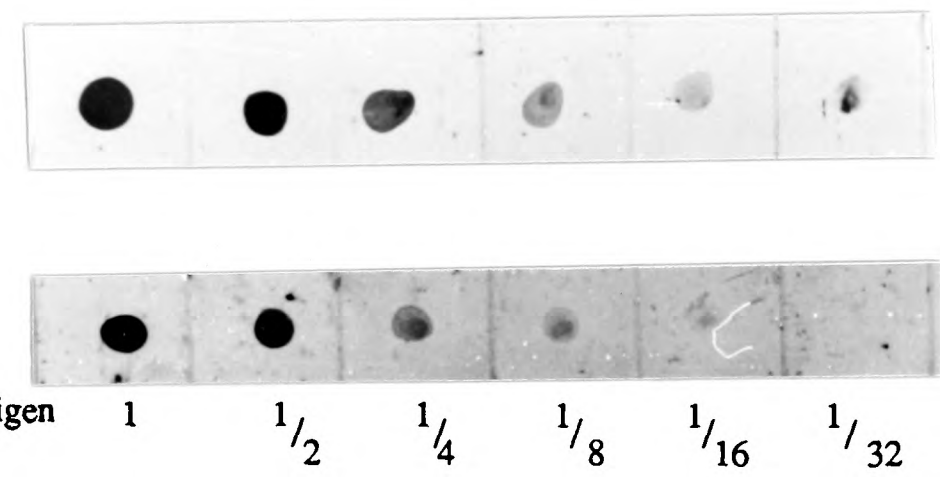


Figure III.2: ELISA of polyclonal antiserum from mice before booster injection.

■ Mouse LE

▲ Mouse NE

◆ Mouse RE

Doubling dilutions of antiserum from 800-fold to 409,600-fold diluted.

In all assays, background (PBSAT only) reading was 0.210 A_{450} units.

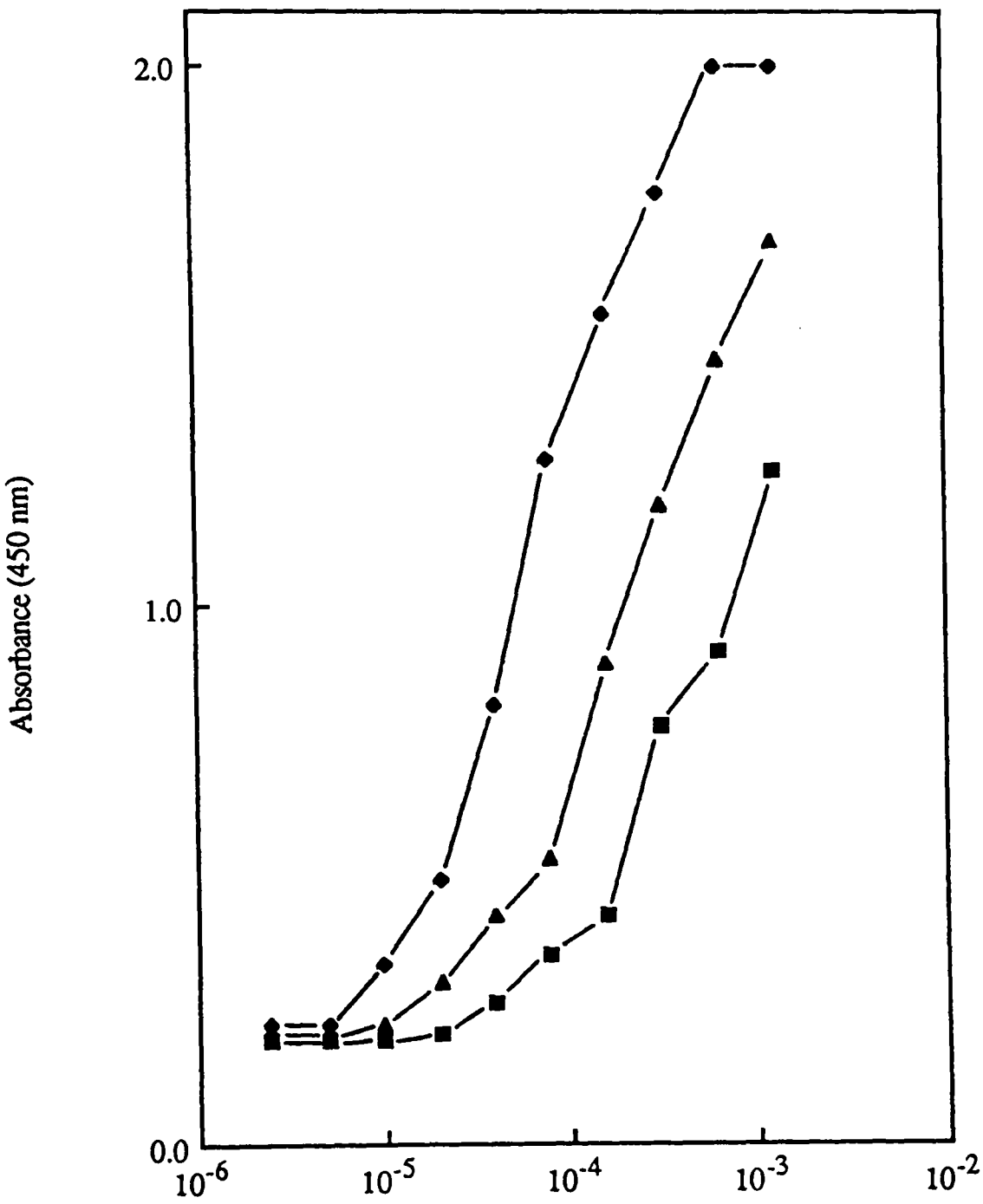
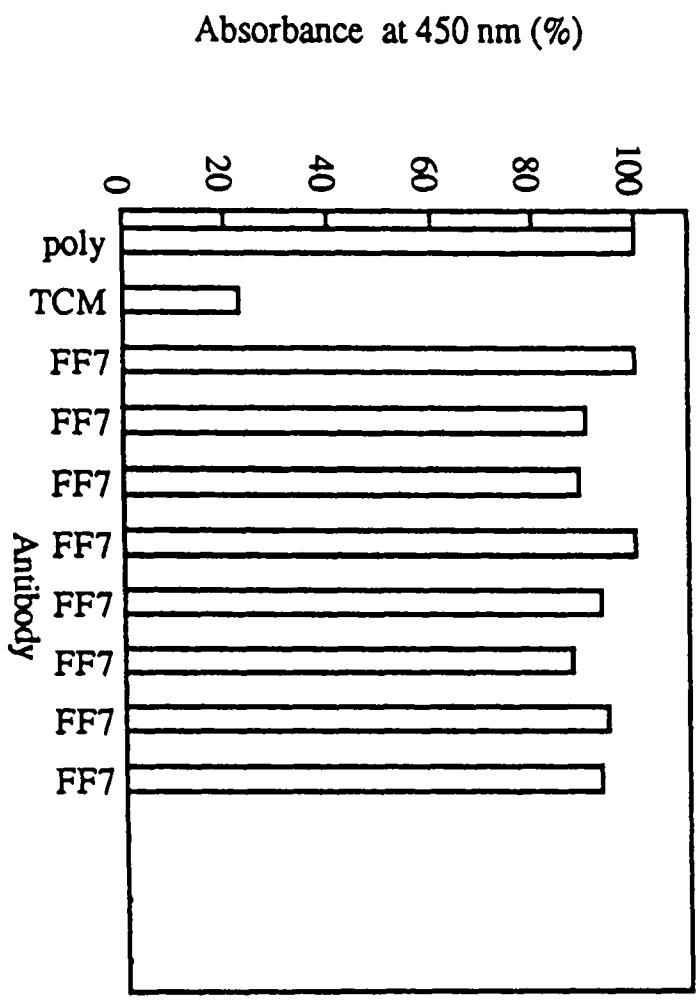
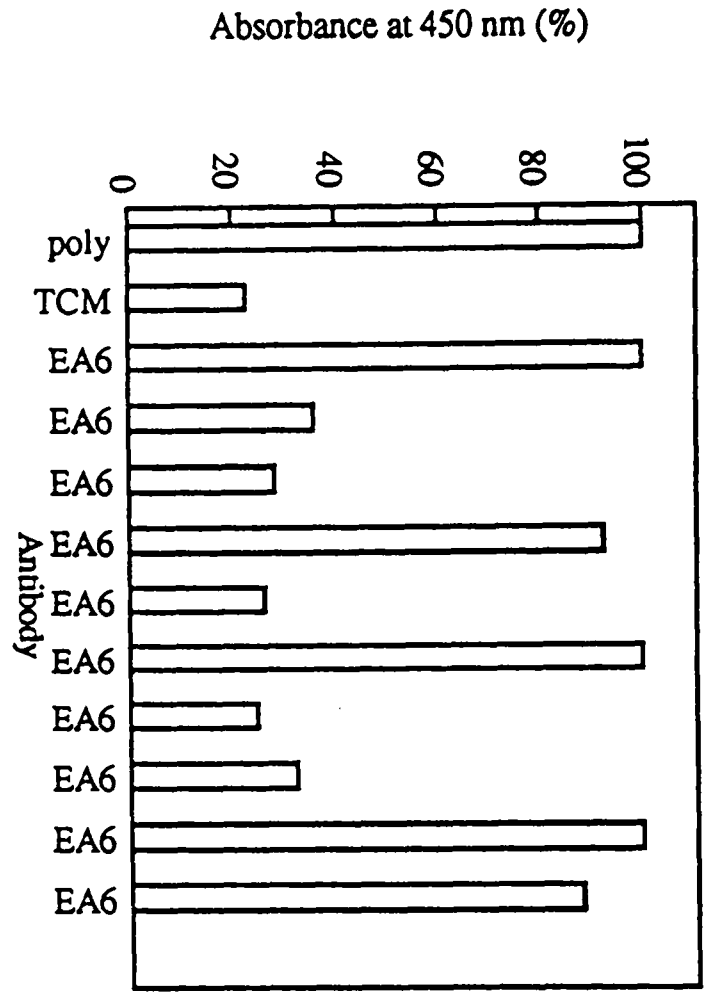

Absorbance (450 nm)
2.0
1.0
0.0
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
Dilution of antiserum

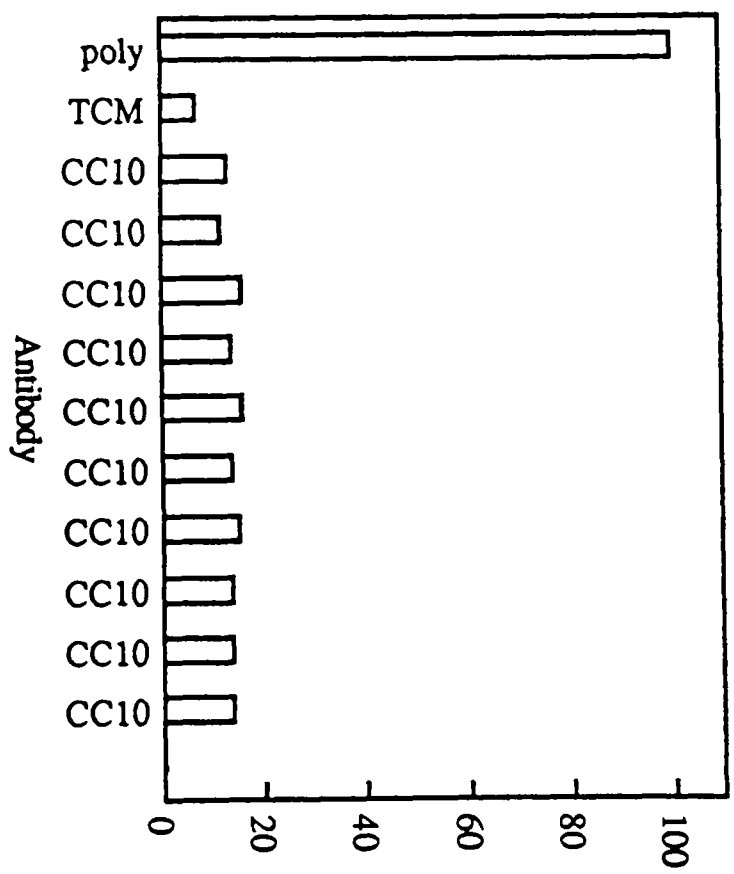
Figure III.3: First stage cloning of hybridomas: 10,000-fold dilution of cells from fusion, assay by ELISA of supernatants from wells containing distinct single colonies.

poly = polyclonal antiserum from mouse NE, 800-fold dilution.

TCM = tissue culture medium containing 20% foetal bovine serum.


Absorbance at 450 nm (%)
0
20
40
60
80
100
poly
TCM
FF7
FF7
FF7
FF7
FF7
FF7
FF7
FF7
Antibody


Absorbance at 450 nm (%)
0
20
40
60
80
100
poly
TCM
EA6
EA6
EA6
EA6
EA6
EA6
EA6
EA6
EA6
Antibody


poly
TCM
CC10
CC10
CC10
CC10
CC10
CC10
CC10
CC10
CC10
Antibody
0
20
40
60
80
100
Absorbance at 450 nm (%)

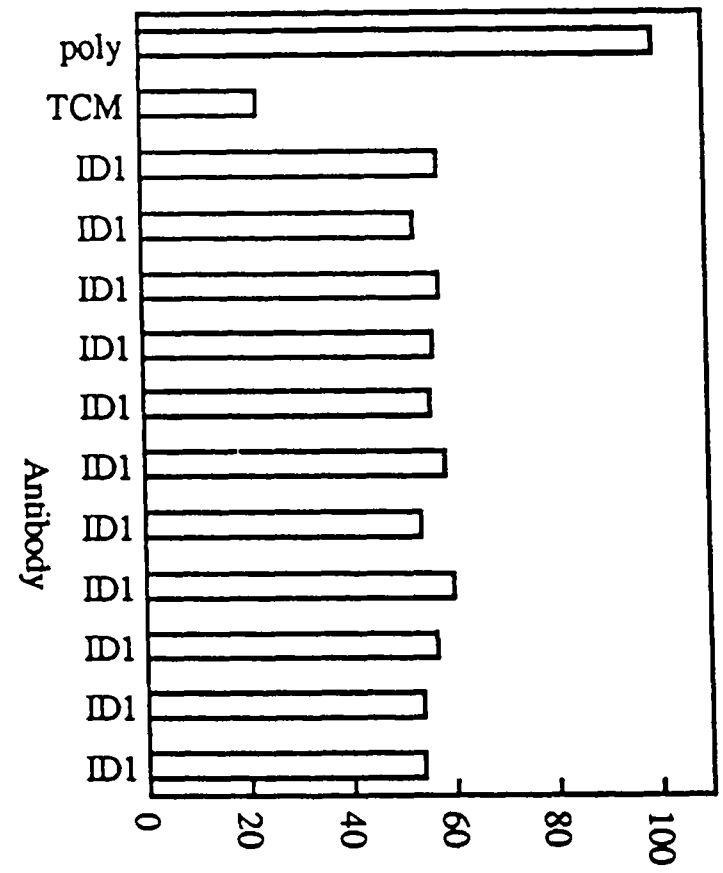

poly
TCM
ID1
ID1
ID1
ID1
ID1
ID1
ID1
ID1
ID1
ID1
Antibody
0
20
40
60
80
100
Absorbance at 450 nm (%)

Figure III.4:Second stage cloning of hybridomas: 5,000-fold dilution of cell line EA6 from first stage cloning, and 50,000-fold dilution of cell line CC10 from first stage cloning.

Assay by ELISA of supernatnats from wells containing distinct single colonies.

poly = polyclonal antiserum from mouse NE, 800-fold diluted.

TCM = tissue culture medium containing 20% foetal bovine serum.

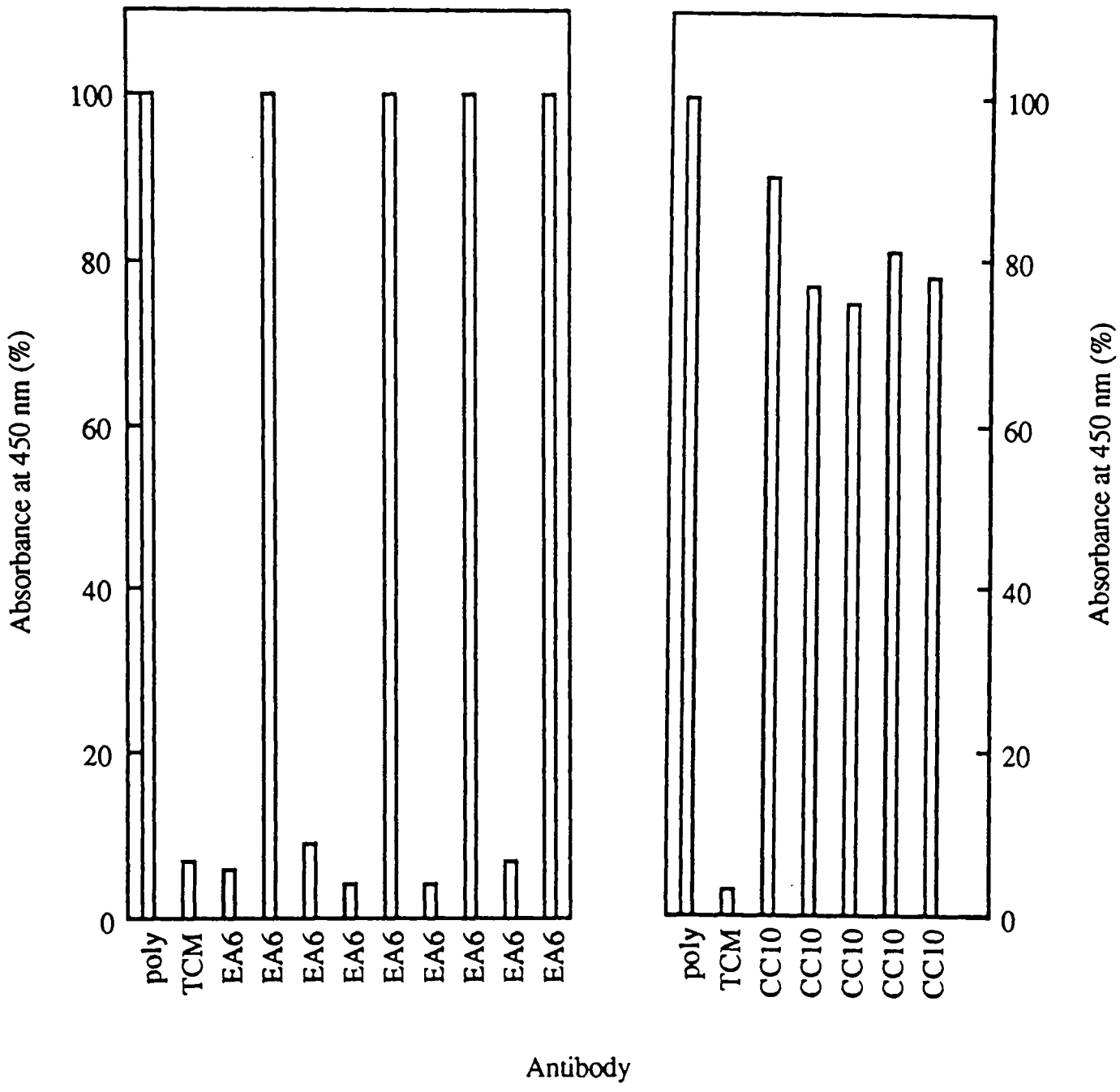

Absorbance at 450 nm (%)
100
80
60
40
20
0
poly
TCM
EA6
EA6
EA6
EA6
EA6
EA6
EA6
EA6
poly
TCM
CC10
CC10
CC10
CC10
CC10
Antibody

Figure III.5: Periodate treatment of antigen-coated ELISA wells and effect on binding of monoclonal antibodies.

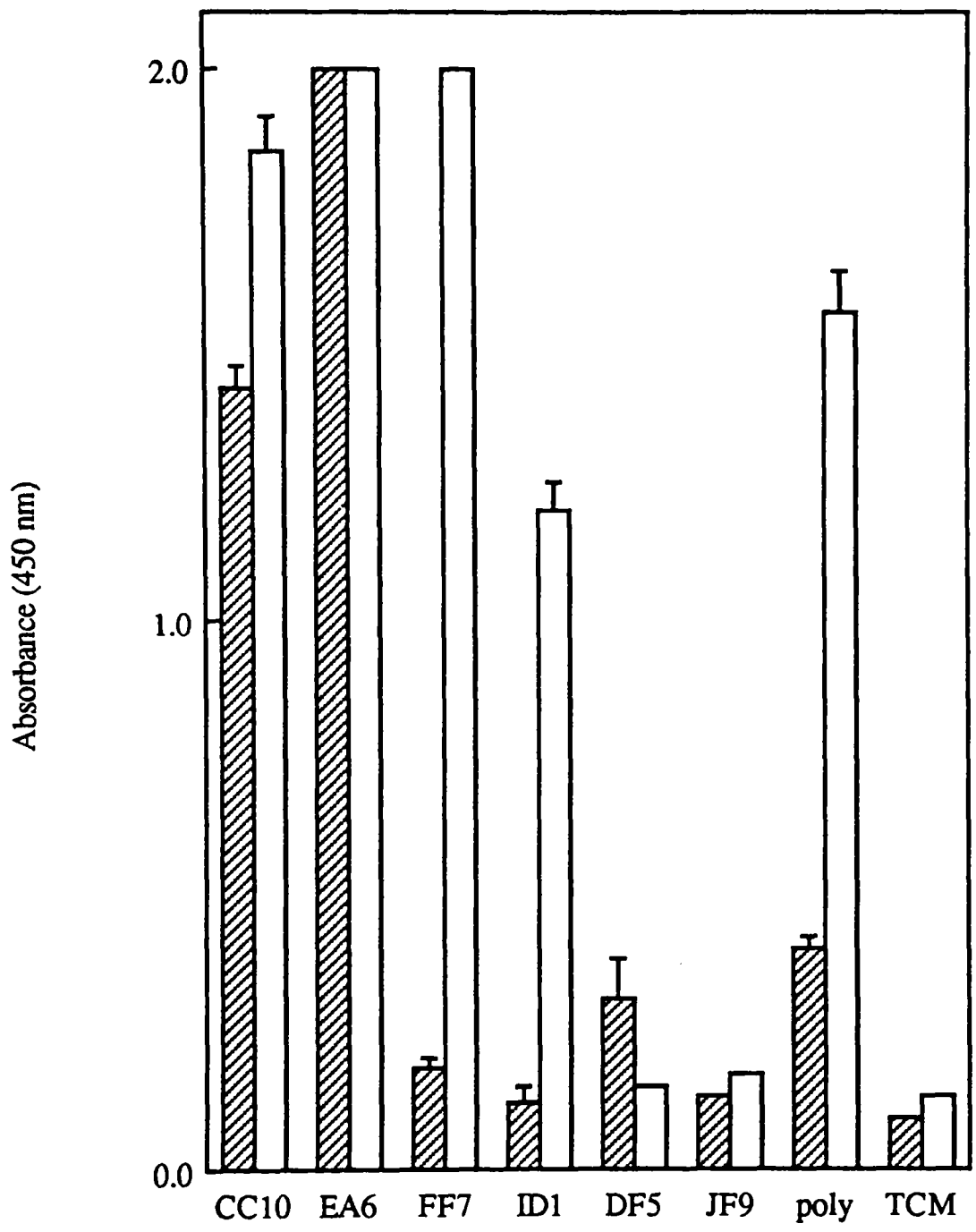
▨ Wells incubated with periodate.

□ Wells incubated with buffer.

Values are means plus or minus one standard deviation (n = 4).

poly = polyclonal antiserum from mouse NE, 800-fold diluted.

TCM = tissue culture medium containing 20% foetal bovine serum.


Absorbance (450 nm)
2.0
1.0
0.0
CC10
EA6
FF7
ID1
DF5
JF9
poly
TCM
Antibody

References

AFFOLTER, H., SIGEL, E. (1979) A simple system for the measurement of ion activities with solvent polymeric membrane electrodes. Analytical Biochem. 97: 315-319

ALLAN, E.F., TREWAVAS, A.J. (1987) The role of calcium in metabolic control. In: The Biochemistry of plants, vol 12, pp 117-149 (STUMPF, P.K., CONN, E.E., Eds.) Academic Press, London, New York.

ALLAN, E., DAWSON, A., DRØBAK, B., ROBERTS, K. (1989) GTP causes calcium release from a plant microsomal fraction. Cellular Signalling 1: 23-29

ALLEN, B.G., KATZ, S., ROUFOGALIS, B.D. (1987) Effects of Ca^{2+}, Mg^{2+} and calmodulin on the formation and decomposition of the phosphorylated intermediate of the erythrocyte Ca^{2+}-stimulated ATPase. Biochem. J. 244: 617-623

ANDERSON, J.M., CHARBONNEAU, H., JONES, M.P. McCANN, R.O., CORMIER, M.J. (1980) Characterisation of the plant NAD kinase activator protein and its identification as calmodulin. Biochemistry 19: 3113-3120

ANDERSON, M.A., SANDRIN, M.S., CLARKE, A.E. (1984) A high proportion of hybridomas raised to a plant extract secrete antibody to arabinose or galactose. Pl.

Physiol. 75: 1013-1016.

ANDREJAUSKAS, E., HERTEL, R., MARMÉ, D. (1985) Specific binding of the calcium antagonist ^{3}H-verapamil to membrane proteins in plants. J. Biol. Chem. 260: 5411-5414

ANTHON, G.E., SPANSWICK, R.M. (1986) Purification and properties of the H^+-translocating ATPase from the plasma membrane of tomato roots. Pl. Physiol. 81: 1080-1085

ASHLEY, C.C., MOISECU, D.G. (1977) Effect of changing the composition of the bathing solutions upon the isomeric tension-$_p$Ca relationship in bundles of crustacean myofibrils.J. Physiol. 270: 627-652

AU, K.S. (1987) Activation of erythrocyte membrane calcium ATPase by calpain. Biochim. Biophys. Acta. 905: 273-278

BADER, H., POST, R.L. (1966) Isolation and characterisation of a phosphorylated intermediate in the (Na^++K^+) system-dependent ATPase. Biochim. Biophys. Acta. 118: 106-115

BANGERTH, F. (1979) Calcium-regulated physiological disorders of plants. Annu. Rev. Phytopathol. 17: 97-122

BARRABIN, H., GARRAHAN, P.J., REGA, A.F. (1980) Vanadate inhibition of the Ca^{2+}-ATPase from human red cell membranes. Biochim. Biophys. Acta 600: 796-804

BATTEY, N.H., VENIS, M.A. (1988) Calcium dependent protein kinase from apple fruit membranes is calmodulin-independent but has calmodulin-like properties. Planta 176: 91-97

BELL, R.M. (1986) Protein kinase C activation by diacylglycerol second messengers. Cell 45: 631-632

BENHAIM, G., CLARKE, A., CARAFOLI, E. (1986) ATPase activity and Ca^{2+}-transport by reconstituted tryptic fragments of the Ca^{2+}-pump of the erythrocyte plasma membrane. Cell Calcium 7: 175-186

BENSADOUN, A., WEINSTEIN, D. (1976) Assay of proteins in the presence of interfering materials. Analytical Biochem. 70: 241-250

BERRIDGE, M.J. (1988a) Inositol trisphosphate and diacylglycerol: two interacting second messengers. Annu. Rev. Biochem. 56: 159-193

BERRIDGE, M.J. (1988b) Inositol lipids and calcium signalling. Proc. Roy. Soc. Lond. Ser. B. 234: 359-378

BERRIDGE, M.J., IRVINE, R.F. (1989) Inositol phosphates and cell signalling. Nature 341: 197-205

BLOWERS, D.P., HETHERINGTON, A., TREWAVAS, A. (1985) Isolation of plasma-membrane-bound calcium/calmodulin-regulated protein kinase from pea using Western blotting. Planta 166: 208-215

BLOWERS, D.P., TREWAVAS, A.J. (1987) Autophosphorylation of plasma-membrane bound calcium-calmodulin dependent protein kinase from pea seedlings and modification of catalytic activity by autophosphorylation. Biochem. Biophys. Res. Commun. 143: 691-696

BLUM, W., HINSCH, K.-D., SCHULTZ, G., WEILER, E.W. (1988) Identification of GTP-binding proteins in the plasma membrane of higher plants. Biochem. Biophys. Res. Commun. 156: 954-959

BLUMWALD, E., POOLE, R.J. (1986) Kinetics of Ca^{2+}/H^{2+} antiport in isolated tonoplast vesicles from storage tissue of *Beta vulgaris*. Plant Physiol. 80: 727-731

BÖGRE, L., OLÁH, Z., DUTIS, D. (1988) Ca^{2+}-dependent protein kinase from alfalfa

(*Medicago varia*): partial purification and autophosphorylation. Plant Sci. 58: 135-44

BOLWELL, G.P., NORTHCOTE, D.H. (1984) Demonstration of a common antigenic site on endomembrane proteins of *Phaseolus vulgaris* by a rat monoclonal antibody. Planta 162: 139-146

BOND, G.H., HUDGINS, P.M. (1980) Inhibition of the red cell Ca^{2+}-ATPase by vanadate. Biochim. Biophys. Acta 600: 781-790

BORKE, J.L., MINAMI, J., VERMA, A., PENNISTON, J.T., KUMAR, R. (1987) Monoclonal antibodies to human erythrocyte membrane Ca^{++}-Mg^{++} adenosine triphosphatase pump recognise an epitope in the basolateral membrane of human kidney distal tubule cells. J. Clin. Invest. 80: 1225-1231

BOSS, W.F., MASSEL, M.O. (1985) Polyphosphoinositides are present in plant tissue culture cells. Biochem. Biophys. Res. Commun. 132: 1018-1023

BOUSTEAD, C.M., SMALLWOOD, M., SMALL, H., BOWLES, D.J., WALKER, J.H. (1989) Identification of calcium-dependent phospholipid-binding proteins in higher plant cells. FEBS Lett. 244: 456-460

BRADLEY, D.J., WOOD, E.A., LARKINS, A.P., GALFRE, G., BUTCHER, G.W., BREWIN, N.J. (1988) Isolation of monoclonal antibodies reacting with peribacteroid membranes and other components of pea root nodules containing *Rhizobium leguminosarum*. Planta 173: 149-160

BRANDT, P., ZURINI, M., NEVE, R.L., RHOADS, R.E., VANAMAN, T.C. (1988) A C-terminal, calmodulin-like regulatory domain from the plasma membrane Ca^{2+}-pumping ATPase. Proc. Natl. Acad. Sci. USA. 85: 2914-2918

BRAUER,D., TU, S.-H. (1989) Phospholipid requirement of the vanadate-sensitive ATPase from maize roots evaluated by two methods. Plant Physiol. 89: 867-874

BREWIN, N.J., ROBERTSON, J.G., WOOD, E.A., WELLS, B., LARKINS, A.P., GALFRE, G., BUTCHER, G.W. (1985) Monoclonal antibodies to antigens in the peribacteroid membrane from *Rhizobium*-induced root nodules of pea cross-react with plasma membranes and Golgi bodies. EMBO. J. 4: 605-611

BRIARS, S.-A., EVANS, D.E. (1989) The calmodulin-stimulated ATPase of maize coleoptiles forms a phosphorylated intermediate. Biochem. Biophys. Res. Commun. 159: 185-191

BRIARS, S.-A., KESSLER, F., EVANS, D.E. (1988) The calmodulin-stimulated ATPase of maize coleoptiles is a 140,000-M_r polypeptide. Planta 176: 283-285

BRISKIN, D.P. (1986) Intermediate reaction states of the red beet plasma membrane ATPase. Arch. Biochem. Biophys. 248: 106-115

BRISKIN, D.P. (1988a) Chemical equivalence of phosphoenzyme reaction states in the catalytic mechanism of the red beet (*Beta vulgaris* L.) plasma membrane ATPase. Plant Physiol. 88: 77-83

BRISKIN, D.P. (1988b) Phosphorylation and dephosphorylation reactions of the red beet plasma membrane ATPase studied in the transient state. Plant Physiol. 88: 84-91

BRISKIN, D.P., LEONARD, R.T. (1982a) Phosphorylation of the adenosine triphosphatase in a deoxycholate-treated plasma membrane fraction from corn roots. Plant Physiol. 70: 1459-1464

BRISKIN, D.P., LEONARD, R.T. (1982b) Partial characterisation of a phosphorylated intermediate associated with the plasma membrane ATPase of corn roots. Proc. Natl. Acad. Sci. USA 79: 6922-6926

BRISKIN, D.P., POOLE, R.J. (1983a) Plasma membrane ATPase of red beet forms a phosphorylated intermediate. Plant Physiol. 71: 507-512

BRISKIN, D.P., POOLE, R.J. (1983b) Evidence for a β-aspartyl phosphate residue in the phosphorylated intermediate of the red beet plasma membrane ATPase. Plant Physiol. 72: 1133-1135

BRISKIN, D.P., THORNLEY, W.R. (1985) Plasma membrane ATPase of sugar beet. Phytochemistry 24: 2797-2802

BROWN, E.G., NEWTON, R.P. (1981) Cyclic AMP and higher plants.Phytochemistry 20: 2453-2461

BUCKHOUT, T.J. (1983) ATP-dependent Ca^{2+} transport in endoplasmic reticulum isolated from roots of *Lepidium sativum.* Planta 159: 84-90

BUCKHOUT, T.J. (1984) Characterisation of a Ca^{2+} transport in purified endoplasmic reticulum membrane vesicles from *Lepidium sativum* L roots. Plant Physiol. 76: 962-967

BUCKHOUT, T.J., MOORE, J.D., YOUNG, K.A., LOW, P.S. (1980) Response of isolated plant membranes to auxins: calcium release.Bot. Gaz. 141: 418-421

BUCKHOUT, T.J., YOUNG, K.A., LOW, P.S., MORRÉ, D.J. (1981) *In vitro* promotion by auxins of divalent ion release from soybean membranes. Plant Physiol. 68: 512-515

BUSH, D.S., BISWAS, A.K., JONES, R.L. (1989) Gibberellic-acid-stimulated Ca^{2+} accumulation in endoplasmic reticulum of barley aleurone: Ca^{2+} transport and steady-state levels.Planta 178: 411-420

BUSH, D.S., JONES, R.L. (1987) Measurement of cytoplasmic calcium in aleurone protoplasts using Indo-1 and Fura-2. Cell Calcium 8: 455-472

BUSH, D.S., SZE, H. (1986) Ca^{2+}-transport in tonoplast and endoplasmic reticulum vesicles isolated from cultured carrot cells. Plant Physiol. 80: 549-555

BUTCHER, R.D., EVANS, D.E. (1987a) Calcium transport by pea root membranes. I. Purification of membranes and characteristics of uptake. Planta 172: 265-278

BUTCHER, R.D., EVANS, D.E. (1987b) Calcium transport by pea root membranes. II. Effects of calmodulin and inhibitors. Planta 172: 273-279

CAMPBELL, A.K. (1983) Intracellular calcium: its universal role as a regulator. John Wiley & Sons, New York

CARAFOLI, E. (1987) Intracellular calcium homeostasis. Annu. Rev. Biochem. 56: 395-433

CARAFOLI, E., ZURINI, M. (1982) The Ca^{2+}-pumping ATPase of plasma membranes: purification, reconstitution and properties. Biochem. Biophys. Acta. 683: 279-301

CARAFOLI, E., NIGGLI, V., PENNISTON, J.T. (1980a) Purification and reconstitution of the calcium-magnesium ATPase of the erythrocyte membrane. Ann. N.Y. Acad. Sci. 358: 159-168

CARAFOLI, E., NIGGLI, V., MALSTRÖM, K., CARONI, P. (1980b) Calmodulin in natural and reconstituted calcium transporting systems. Ann. N. Y. Acad. Sci. 356: 258-266

CARAFOLI, E., ZURINI, M., NIGGLI, V., KREBS, J. (1982) The calcium transporting ATPase of erythrocytes. Ann. N.Y. Acad. Sci. 402: 304-328

CARIDE, A.J., GORSKI, J.P., PENNISTON, J.T. (1988) Topology of the erythrocyte Ca^{2+} pump: a monoclonal antibody against the almost inaccessible extracellular face. Biochem. J. 255: 663-670

CARTER, S.G., TIPTON, C.L. (1987) A high affinity (Ca^{2+}+Mg^{2+})-ATPase from *Zea mays* microsomes. Phytochemistry 26: 2191-2196

CHAFOULEAS, J.G., DEDMAN, J.R., MUNJAAL, R.P., MEANS, A.R. (1979)

Calmodulin: development and application of a sensitive radioimmunoassay. J. Biol. Chem. 254: 10262-10267

CHIATANTE, D., LEVI, M., SGORBATI, S., SPARVOLI, E. (1987) Protein kinase activity in meristematic root tips of *Pisum sativum* during germination. Plant Sci. 53: 271-279

CHIN, J.J.C. (1982) Monoclonal antibodies that immunoreact with a cation-stimulated plant membrane ATPase. Biochem. J. 203: 51-54

CHOQUETTE, D., HAKIM, G., FILOTEO, A.G., PLISHKER, G.A., BOSTWICK, J.R., PENNISTON, J.T. (1984) Regulation of plasma membrane calcium ATPase by lipids of the phosphatidylinositol cycle. Biochem. Biophys. Res. Commun. 125: 908-915

CHURCHILL, K.A., HOLAWAY, B., SZE, H. (1983) Separation of two types of electrogenic H^+-pumping ATPases from oat roots. Plant Physiol. 73: 921-928

CLEMENT, J.D., GHISLAIN, M., DUFOUR, J.P., SCALLA, R. (1986) Immunodetection of a 90,000 M_r polypeptide related to yeast plasma membrane ATPase in plasma membrane from maize shoots. Plant Sci. 45: 43-50

CLEVELAND, D.W. (1983) Peptide mapping in one dimension by limited proteolysis of sodium dodecyl sulphate-solubilised proteins. Meth. Enzymol. 96: 222-229

COCUCCI, M., NEGRINI, N. (1988) Changes in levels of calmodulin and of a calmodulin inhibitor in the early phases of radish (*Raphanus sativus* L.) seed germination. Plant Physiol. 88: 910-914

COHEN, J.D., NADLER, K.D. (1976) Calcium requirement for indoleacetic-acid induced acidification by *Avena* coleoptiles. Plant Physiol. 57: 347-350

COHEN, P. (1988) Protein phosphorylation and hormone action. Proc. Roy. Soc.

Lond. Ser. B. 234: 115-144

DAWSON, A.P. (1985) GTP enhances inositol trisphosphate-stimulated Ca^{2+} release from rat liver microsomes. FEBS Lett. 185: 147-150

DAWSON, A.P., COMERFORD, J.G., FULTON, D.V. (1986) The effect of GTP on inositol 1,4,5-trisphosphate-stimulated Ca^{2+} efflux from a rat liver microsomal fraction. Biochem. J. 234: 311-315

DAWSON, A.P., HILLS, J.G., COMERFORD, J.G. (1987) The mechanism of action of GTP on Ca^{2+} efflux from rat liver microsomal vesicles. Biochem. J. 244: 87-92

De La FUENTE, R.K., LEOPOLD, A.C. (1973) A role for calcium in auxin transport. Plant Physiol. 51: 845-847

DE SILVA, D.L.R., HETHERINGTON, A.M., MANSFIELD, T.A. (1985a) Synergism between Ca^{2+} and abscisic acid in preventing stomatal opening. New Phytol. 100: 473-482

DE SILVA, D.L.R., COX, R.C., HETHERINGTON, A.M., MANSFIELD, T.A. (1985b) Suggested involvement of calcium and calmodulin in the responses of stomata to abscisic acid. New Phytol. 101: 555-563

DEWEY, F.M., BRASIER, C.M. (1988) Development of ELISA for *Ophiostoma ulmi* using antigen-coated wells. Plant Pathology 37: 28-35

DIETER, P. (1984) Calmodulin and calmodulin-mediated processes in plants. Plant, Cell and Env. 7: 371-380

DIETER, P., MARMÉ, D. (1980a) Calmodulin-activated plant microsomal calcium uptake, and purification of plant NAD kinase and other proteins by calmodulin-sepharose affinity chromatography. Ann. N.Y. Acad. Sci. 356: 371-373

DIETER, P., MARMÉ, D. (1980b) Partial purification of plant NAD kinase by

calmodulin-sepharose affinity chromatography. Cell Calcium 1: 279-286

DIETER, P., MARMÉ, D. (1980c) Ca^{2+}-transport in mitochondrial and microsomal fractions from higher plants. Planta 150: 1-8

DIETER, P., MARMÉ, D. (1980d) Calmodulin activation of plant microsomal Ca^{2+} uptake. Proc. Natl. Acad. Sci. USA 77: 7311-7314

DIETER, P., MARMÉ, D. (1981a) Far-red light irradiation of intact corn seedlings affects mitochondrial and calmodulin-dependent microsomal Ca^{2+} transport. Biochem. Biophys. Res. Commun. 101: 749-755

DIETER, P., MARMÉ, D. (1981b) A calmodulin-dependent, microsomal ATPase from corn (*Zea mays*). FEBS Lett. 125: 245-248

DIETER, P., MARMÉ, D. (1983) The effect of calmodulin and far-red light on the kinetic properties of the mitochondrial and microsomal Ca^{2+} transport system from corn. Planta 159: 277-281

DILLENSCHNEIDER, M., HETHERINGTON, A., GRAZIANA, A., ALIBERT, G., BERTA, P., HAIECH, J., RANJEVA, R. (1986) The formation of inositol phosphate derivatives by isolated membranes from *Acer pseudoplatanus* is stimulated by guanine nucleotides. FEBS Lett. 208: 413-17

DRØBAK, B.K., FERGUSON, I.B. (1985) Release of Ca^{2+} from plant hypocotyl microsomes by inositol-1,4,5 trisphosphate. Biochem. Biophys. Res. Comm. 130: 1241-1246

DRØBAK, B.K., FERGUSON, I.B., DAWSON, A.P., IRVINE, R.F. (1988a) Inositol-containing lipids in suspension-cultured plant cells. Plant Physiol. 87: 217-222

DRØBAK, B.K., ALLAN, E.F., COMERFORD, J.G., ROBERTS, K., DAWSON, A.P. (1988b) Presence of guanine nucleotide-binding proteins in a plant hypocotyl microsomal fraction. Biochem. Biophys. Res. Commun. 150: 889-903

DUPONT, F.M., LEONARD, R.T. (1980) Solubilisation and partial purification of the adenosine triphosphatase from a corn root plasma membrane fraction. Plant Physiol. 65: 931-938

ELLIOT, D.C. (1980) Calmodulin inhibitor prevents plant hormone response. Biochem. Int. 1: 290-294

ELLIOT, D.C. (1983) Inhibition of cytokinin-regulated responses by calmodulin binding compounds. Plant Physiol. 72: 215-218.

ELLIOT, D.C., BATCHELOR, S.M., CASSAR, R.A., MARINOS, N.G. (1983) Calmodulin-binding drugs affect responses to cytokinin, auxin and gibberellic acid. Plant Physiol. 72: 219-224

ELLIOT, D.C., FOURNIER, A., KOKKE, Y.S. (1988) Phosphatidylserine activation of plant protein kinase C. Phytochemistry. 27: 3725-3730

ELLIOT, D.C., KOKKE, Y.S. (1987) Partial purification and properties of a protein kinase C type enzyme from plants. Phytochemistry. 26: 2929-2935

ELLIOT, D.C., SKINNER, J.D. (1986) Calcium-dependent phospholipid-activated protein kinase in plants. Phyochemistry 25: 39-44

ETTLINGER, C., LEHLE, L. (1988) Auxin induces rapid changes in phosphatdylinositol metabolites. Nature 331: 176-178

EVANS, D.E. (1988) Regulation of cytoplasmic free calcium by plant cell membranes. Cell Biol. Int. Rep. 12: 383-396

EVANS, P.T., HOLAWAY, B.L., MALMBERG, R.L. (1988) Biochemical differentiation in the tobacco flower probed with monoclonal antibodies. Planta 175: 259-269

EVANS, W.H. (1987) Organelles and membranes of animal cells. In: Biological membranes: a practical approach, pp 1-36 (FINDLAY, J.B.C., EVANS, W.H., Eds.) IRL

Press, Oxford, Washington

FEIZI, T., CHILDS, R.A. (1987) Carbohydrates as antigenic determinants of glycoproteins. Biochem. J. 245: 1-11

FELLE, H. (1988a) Auxin causes oscillations of cytosolic free calcium and pH in *Zea mays* coleoptiles. Planta 174: 495-499

FELLE, H. (1988b) Cytoplasmic free calcium in *Riccia fluitans* L. and *Zea mays* L.: interaction of Ca^{2+} and pH ? Planta 176: 248-255

FELLER, K. (1989) Characterisation of a protein kinase from soybean seedlings. Study of the variation of calcium-regulated kinase activity during infection with the incompatible race 1 and the compatible race 3 of *Phytophthora megasperma* f.sp. *glycinea* by *in vitro* phosphorylation of calf thymus histone H1. Plant Sci. 60: 67-75

FERGUSON, I.B. (1984) Calcium in plant senescence and fruit ripening. Plant, cell Environ. 7: 477-489

FILOTEO, A.G., GORSKI, J.P., PENNISTON, J.T. (1987) The ATP-binding site of the erythrocyte membrane Ca^{2+} pump. Amino acid sequence of the fluorescein isothiocyanate-reactive region. J. Biol. Chem. 262: 6526-6530

FITTER, M.S., NORMAN, P.M., HAHN, M.G., WINGATE, V.P.M., LAMB, C.J. (1987) Identification of somatic hybrids in plant protoplast fusions with monoclonal antibodies to plasma-membrane antigens. Planta 170: 49-54

FODER, B., SCHARFF, O. (1981) Decrease of apparent calmodulin affinity of erythrocyte (Ca^{2+}+Mg^{2+})-ATPase at low Ca^{2+} concentrations. Biochim. Biophys. Acta. 649: 367-376

FOUNG, S.K.H., SASAKI, D.T., GRUMET, F.C., ENGLEMAN, E.C. (1982) Production of functional human T-T hybridomas in selection medium lacking aminopterin and

thymidine. Proc. Natl. Acad. Sci. USA 79: 7484-7488

FUKUMOTO, M., VENIS, M.A. (1986) ATP dependent Ca^{2+} transport in tonoplast vesicles from apple fruit. Plant Cell Physiol. 27: 491-497

FUTAI, M., NOUMI, T., MAEDA, M. (1989) ATP synthase (H^+-ATPase): results by combined biochemical and molecular biological approaches. Annu. Rev. Biochem. 58: 111-136

GALFRE, G., MILSTEIN, C. (1981) Preparation of monoclonal antibodies: strategies and procedures. Methods Enzymol. 73: 3-46

GALLAGHER, S.R., LEONARD, R.T. (1982) Effect of vanadate, molybdate and azide on membrane-associated ATPase and solubule phosphatase activities of corn.Plant Physiol. 70: 1335-1340

GALLAGHER, S.R., LEONARD, R.T. (1987) Electrophoretic characterisation of a detergent-treated plasma membrane fraction from corn roots. Plant Physiol. 83: 265-271

GEHRING, U. (1987) Steriod hormone receptors: biochemistry, genetics and molecular biology. Tr. Biochem. Sci. 12: 399-402

GIANNINI, J.L., GILDENSOPH, L.H., REYNOLDS-NIESMAN, I., BRISKIN, D.P. (1987a) Calcium transport in sealed vesicles from red beet (*Beta vulgaris* L.) storage tissue: I.characterisation of a Ca^{2+} pumping ATPase associated with the endoplasmic reticulum. Plant Physiol. 85: 1129-1136

GIANNINI, J.L., RUIZ-CRISTIN, J., BRISKIN, D.P. (1987b) Calcium transport in sealed vesicles from red beet (*Beta vulgaris.* L.) storage tissue: II.characterisation of $^{45}Ca^{2+}$ uptake into plasma membrane vesicles. Plant Physiol. 85: 1137-1142

GIESOW, M.J., WALKER, J.H., BOUSTEAD, C., TAYLOR, W. (1987) Annexins -

new family of Ca^{2+}-regulated phospholipid binding protein. Bioscience Rep. 7: 287-298

GIETZEN, K., KOLANDT, J. (1985) Antibodies against erythrocyte Ca^{2+} -transport ATPase specifically inhibit the calmodulin-dependent fraction of the enzyme's activity. Biochem. J. 228: 479-485

GIETZEN, K., TEJCKA, M., WOLF, H.U. (1980) Calmodulin affinity chromatography yields a functional purified erythrocyte (Ca^{2+}+Mg^{2+})-dependent ATPase.Biochem. J. 189: 81-88

GILMAN, A.G. (1987) G-proteins: transducers of receptor generated signals. Annu. Rev. Biochem. 56: 615-649

GILROY, S., HUGHES, W.A., TREWAVAS, A.J. (1986) The measurement of intracellular calcium levels in protoplasts from higher plant cells. FEBS Lett. 199: 217-221

GILROY, S., BLOWERS, D.P., TREWAVAS, A.J. (1987) Calcium: a regulation system emerges in plant cells (review). Development 100: 181-184

GILROY, S., HUGHES, W.A., TREWAVAS, A.J. (1989) A comparison between Quin-2 and Aequorin as indicators of cytoplasmic calcium levels in higher plant cell protoplasts. Plant Physiol. 90: 482-491

GODING, J.W. (1983) Monoclonal antibodies: principles and practice. Academic Press, London, New York.

GOLDBERG, N.D., HADDOX, M.K. (1977) Cyclic GMP metabolism and involvement in biological regulation. Annu. Rev. Biochem. 46: 823-896

GOPINATH, R.M., VINCENZI, F.F. (1977) Phosphodiesterase protein activator mimics red blood cell cytoplasmic activator of (Ca^{2+}-Mg^{2+}) ATPase. Biochem. Biophys. Res. Commun. 77: 1203-1209

GÖRG, A., POSTEL, W., WESER, J., SCHIWARA, H.W., BOESKEN, W.H. (1985)

Horizontal SDS electrophoresis in ultrathin pore-gradient gels for the analysis of urinary proteins. Science Tools 32: 5-9

GRAF, E., VERMA, A.K., GORSKI, J.P., LOPASCHUK, G., NIGGLI, V., ZURINI, M., CARAFOLI, E., PENNISTON, J.T. (1982) Molecular properties of Ca^{2+}-pumping ATPase from human erythrocytes. Biochemistry 21: 4511-4516

GRÄF, P., WEILER, E.W. (1989) ATP-driven transport in sealed plasma membrane vesicles prepared by aqueous two-phase partitioning from leaves of *Commelina communis*. Physiol. Plant. 75: 469-478

GRAZIANA, A., FOSSET, M., RANJEVA, R., HETHERINGTON, A.M., LAZDUNSKI, M. (1988) Ca^{2+} channel inhibitors that bind to plant cell membranes block Ca^{2+} entry into protoplasts. Biochemistry 27: 764-768

GROSS, J. (1982) Oxalate-enhanced active calcium uptake in membrane fractions from zucchini squash. In: Plasmalemma and tonoplast: their Function in the Plant Cell, pp 369-376 (MARMÉ, D., MARRÉ, E., HERTEL, R., Eds.) Elsevier Biomedical, Amsterdam.

GROSS, J., MARMÉ, D. (1978) ATP-dependent calcium uptake into plant membrane vesicles. Proc. Natl. Acad. Sci. USA 75: 1232-1236

GROSS, J., AYADI, A., MARMÉ, D. (1979) Protochlorophyll(ide)$_{630}$ photosensitises active Ca^{2+} accumulation in microsomal and mitochondrial fractions isolated from plants. Photochemistry and photobiology 30: 615-621

GUILFOYLE, T.J. (1986) Auxin-regulated gene expression in higher plants. Crit. Rev. Plant Sci. 4: 247-276

GUTTMANN, R.D. (Ed.) (1981) Immunology. The Upjohn Company, Michigan.

HAHN, M.G., LERNER, D.R., FITTER, M.S., NORMAN, P.M., LAMB, C.J. (1987)

Characterisation of monoclonal antibodies to protoplast membranes of *Nicotiana tabacum* identified by an enzyme-linked immunosorbent assay. Planta 171: 453-465

HALL, J.L. (1983) Plasma membranes. In: Isolation of membrane organelles from plant cells, pp 55-81 (HALL, J.L. and MOORE, A.L. Eds.) Academic Press, London, New York.

HAMES, B.D., RICKWOOD, D. (1983) Gel electrophoresis of proteins: a practical approach. IRL Press, Oxford, Washington.

HANAHAN, D.J. (1986) Platelet activating factor: a biologically active phosphoglyceride. Annu. Rev. Biochem. 55: 483-509

HARLOW, E., LANE, D. (1988) Antibodies: a laboratory manual. Cold Spring Harbour Laboratory.

HARMON, A.C., PUTNAM-EVANS, C., CORMIER, M.J. (1987) A calcium-dependent but calmodulin-independent protein kinase from soybean. Plant Physiol. 83: 830-837

HASSELBACH, H., WAAS, W. (1982) Energy coupling in sarcoplasmic reticulum Ca^{2+} transport: an overview. Ann. N.Y. Acad. Sci. 402: 459-469

HEIM, S., WAGNER, K.G. (1986) Evidence of phosphorylated phosphatdylinositols in the growth cycle of suspension cultured plant cells. Biochem. Biophys. Res. Commun. 134: 1175-1181

HEIM, S., WAGNER, K.G. (1987) Enzymatic activities of the phosphatidylinositol cycle during growth of suspension cultured plant cells. Plant Sci. 49: 167-173

HEIM, S., BAULEKE, A., WYLEGALLA, C., WAGNER, K.G. (1987) Evidence of phosphatidlyinositol and diacylglycerol kinases in suspension cultured plant cells. Plant Sci. 49: 159-165

HENRY, H., PILET, P.E. (1988) Inhibition by vanadate of the tonoplast H^+ translocating ATPase of *Rubus* cells. Plant Sci. 56: 149-154

HEPLER, P.K. (1986) Calcium regulation of mitosis: the metaphase/anaphase transition. In: Molecular and cellular aspects of calcium in plant development, pp 167-174 (TREWAVAS, A.J., Ed.) NATO ASI Series, vol 104. Plenum Press, New York, London.

HEPLER, P.K., WAYNE, R.O. (1985) Calcium and plant development. Annu. Rev. Plant Physiol. 36: 397-439

HETHERINGTON, A., TREWAVAS, A. (1982) Calcium-dependent protein kinase in pea shoot membranes. FEBS Lett. 145: 67-71

HETHERINGTON, A.M., TREWAVAS, A.J. (1984a) Activation of a pea membrane protein kinase by calcium ions. Planta 161: 409-417

HETHERINGTON, A.M., TREWAVAS, A.J. (1984b) Binding of nitrendipine, a calcium channel blocker, to pea shoot membranes. Plant Sci. Lett. 35: 109-113

HODGES, T.K., LEONARD, R.T., BRACKER, C.E., KEENAN, T.W. (1972) Purification of an ion-stimulated adenosine triphosphatase from plant roots: association with plasma membrane. Proc. Natl. Acad. Sci. USA 69: 3307-3311

HODGES, T.K., LEONARD, R.T. (1974) Purification of a plasma-membrane bound adenosine triphosphatase from plant roots. Methods Enzymol 32: 392-406

IRVINE, R.F., LETCHER, A.J., DAWSON, R.M.C. (1980) Phosphatidylinositol phosphodiesterase in higher plants. Biochem. J. 192: 279-283

IRVINE, R.F., LETCHER, A.J., LANDER, D.J., DRØBAK, B.K., DAWSON, A.P., MUSGRAVE, A. (1989) Phosphatidylinositol(4,5)bisphosphate and phosphatidylinositol(4)phosphate in plant tissues. Plant Physiol. 89: 888-892

JACOBS, M., THELEN, M.P., FARNDALE, R.W., ASTLE, M.C., RUBERY, P.H.

(1988) Specific guanine nucleotide binding by membranes from *Cucurbita pepo* seedlings. Biochem. Biophys. Res. Commun. 155: 1478-1484

JAFFÉ, L.F. (1980) Calcium explosions as triggers of development.Ann. N.Y. Acad. Sci. 339: 86-101

JAMES, P., ZVARITCH, E.I., SHAKHPARONOV, M.I., PENNISTON, J.T., CARAFOLI, E. (1987) The amino acid sequence of the phosphorylation domain of the erythrocyte Ca^{2+} ATPase Biochem. Biophys. Res. Commun. 149: 7-12

JAMES, P., MAEDA, M., FISHER, R., VERMA, A.K., KREBS, J., PENNISTON, J.T., CARAFOLI, E. (1988) Identification and primary structure of a calmodulin binding domain of the Ca^{2+} pump of human erythrocytes. J. Biol. Chem. 263: 2905-2910

JARRETT, H.W., PENNISTON, J.T. (1977) Partial purification of the Ca^{2+} -Mg^{2+} ATPase activator from human erythrocytes: its similarity to the activator of 3':5'-cyclic nucleotide phosphodiesterase. Biochem. Biophys. Res. Commun. 77: 1210-1216

JEFFERY, D.A., ROUFOGALIS, B.D., KATZ, S. (1981) The effect of calmodulin on the phosphoprotein intermediate of Mg^{2+}-dependent Ca^{2+}-stimulated adenosine triphosphatase in human erythrocyte membranes. Biochem. J. 194: 481-486.

KAGAWA, Y., RACKER, E. (1971) Partial resolution of the enzymes catalyzing oxidative phosphorylation.XXV: Reconstitution of vesicles catalysing ^{32}Pi-adenosine triphosphate exchange.J. Biol. Chem. 240: 5477-5487

KASAMO, K. (1986) Comparison of plasma membrane and tonoplast H^+-translocating ATPases in *Phaseolus mungo* roots.Plant Cell Physiol. 27: 49-59

KASAMO, K. (1987) Reconstitution and characterisation of H^+-translocating ATPase from the plasma membrane of *Phaseolus mungo* roots. Plant Cell Physiol 28: 19-28

KATZ, S., BLOSTEIN, R. (1975) Ca^{2+}-stimulated membrane phosphorylation and AT-Pase activity of the human erythrocyte. Biochem. Biophys. Acta. 389: 314-324

KAUSS, H. (1987) Some aspects of calcium-dependent regulation in plant metabolism. Annu. Rev. Plant Physiol. 38: 47-72.

KEITH, C.H., RATEN, R., MAXFIELD, F.R., BAJER, A., SELANSKI, M.L. (1985) Local cytoplasmic calcium gradients in living mitotic cells. Nature 316: 848-850

KENNET, R.H., DAVIS, K.A., TUNG, A.S., KILMAN, N.R. (1978) Hybrid plasmocytoma production: fusion with adult spleen cells, monoclonal spleen fragments, neotonal spleen cells and human spleen cells. Curr. Top. Microbiol. Immunol. 81: 77-91

KEY, G., WEILER, E.W. (1988) Monoclonal antibodies identify common and differentiation-specific antigens on the plasma membrane of guard cells of *Vicia faba* L. Planta 176: 472-481

KLEE, C.B. (1980) Calmodulin: structure-function relationships. In: Calcium and cell function, vol I, pp 59-78 (CHEUNG, W.Y., Ed.) Academic Press, London, New York.

KLUCIS, E., POLYA, G.M. (1987) Calcium-independent activation of two plant leaf calcium-regulated protein kinases by unsaturated fatty acids. Biochem. Biophys. Res. Commun. 147: 1041-1047

KLUCIS, E., POLYA, G.M. (1988) Localisation, solubilisation and characterisation of plant membrane-associated calcium-dependent protein kinases. Plant Physiol. 88: 164-171

KNAUF, P.A., PROVERBIO, F., HOFFMAN, J.F. (1974) Electrophoretic separation of different phosphoproteins associated with Ca-ATPase and Na, K-ATPase in human red cell ghosts. J. Gen. Physiol. 63: 324-336

KÖHLER, G., MILSTEIN, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256: 495-497

KUBOWICZ, B.D., VAN DER HOEF, L.N., HANSON, J.B. (1982) ATP-dependent Ca^{2+}-transport in plasma membrane fractions from soybean hypocotys. Plant Physiol. 69: 187-191

KUHLEMEIER, C., GREEN, P.J., CHUA, N. (1987) Regulation of gene expression in higher plants. Annu. Rev. Plant Physiol. 38: 221-257

LAEMMLI, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bactenophage T4. Nature 227: 680-685

LAMBERT, A.-M., VANTARD, M. (1986) Calcium and calmodulin as regulators of chromosome movement during mitosis in higher plants. In: Molecular and cellular aspects of calcium in plant development, pp 175-184(TREWAVAS, A.J., Ed.) NATO ASI Series vol. 104, Plenum Press, New York, London.

LANE, D., KOPROWSKI, H. (1982) Molecular recognition and the future of monoclonal antibodies. Nature 296: 200-202

LEE, K.S., TSIEN, R.W. (1983) Mechanism of calcium channel blockade by verapamil, D600, diltiazem and nitrendipine in single dialysed heart cells. Nature 302: 790-794

LEIDGENS, W., GRÜTZMANN, R., SCHNEIDER, H.A.W. (1980) Highly efficient purification of the labile plant enzyme 5-aminolevulinate dehydratase (EC 4.2.1.24) by means of monoclonal antibodies. Z. Naturforsch 35c: 958-962

LEMBI, C., MORRÉ, D.J., St-THOMPSON, K., HERTEL, R. (1971) N-1-Napthylphthalamic acid binding activity of a plasma membrane-rich fraction from maize coleoptiles. Planta 99: 37-45

LEONARD, R.T., HANSEN, D., HODGES, T.K. (1973) Membrane-bound adenosine

triphosphatase activities of oat roots. Plant Physiol. 51: 749-754

LEVINE, B.A., WILLIAMS, R.J.P. (1982) The chemistry of calcium ion and its biological relevance.In: The role of calcium in biological systems, vol I, pp 67-91 (ANGHILER, L.J., TUFFET-ANGHILERI, A.M., Eds.) CRC Press, Boca Raton, Florida, USA.

LEW, R.R., BRISKIN, D.P., WYSE, R.E. (1986) Ca^{2+} uptake by endoplasmic reticulum from zucchini hypocotyls.Plant Physiol. 82: 47-53

LICHTNER, R., WOLF, H.U. (1980a) Phosphorylation of the isolated high affinity (Ca^{2+}+Mg^{2+})-ATPase of the human erythrocyte membrane. Biochim. Biophys. Acta 598: 472-485

LICHTNER, R., WOLF, R.U. (1980b) Charaterisation of the phosphorylated intermediate of the isolated high affinity (Ca^{2+}+Mg^{2+})-ATPase of human erythrocyte membranes. Biochim. Biophys. Acta 598: 486-493

LING, V., ZIELINSKI, R.E. (1989) Cloning of cDNA sequences encoding the calcium-binding protein, calmodulin, from barley (*Hordeum vulgare* L.) Plant Physiol. 90: 714-719

LIPMAN. F., TUTTLE, L.C. (1945) A specific micromethod for the determination of acylphosphates. J. Biol. Chem. 159: 21-28

LOBLER, M., KLAMBT, D. (1985a) Auxin binding protein from coleoptile membranes of corn: I. Purification by immunological methods and characterisation. J. Biol. Chem. 260: 9848-9853

LOBLER, M., KLAMBT, D. (1985b) Auxin binding protein from coleoptile membranes of corn: II. Localisation of a putative auxin receptor. J. Biol. Chem. 260: 9854-9859

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L., RANDALL, R.J. (1951) Protein measurement with the folin phenol reagent.J. Biol. Chem. 193: 265-275

LUCANTONI, A., POLYA, G.M. (1987) Activation of wheat embryo calcium-regulated protein kinase by unsaturated fatty acids in the presence and absence of calcium.FEBS Lett. 22: 33-36

LUTERBACHER, S., SCHATZMAN, H.J. (1983) The site of action of La^{3+} in the reaction cycle of the human red cell membrane Ca^{2+} pump ATPase.Experientia 39: 311-312

LYNCH, T.J., CHEUNG, W.Y. (1979) Human erythrocyte Ca^{2+}+Mg^{2+} ATPase: mechanism of stimulation by Ca^{2+}. Arch. Biochem. Biophys. 194: 165-170

MACDONALD, M.M., DUNSTAN, R.H., DEWEY, F.M. (1989) Detection of low M_r glycoproteins in surface washings of some fungal cultures by gel-filtration HPLC and by monoclonal antibodies. J. Gen. Microbiol. 135: 375-383

MACKLON, A.E.S. (1984) Calcium fluxes at the plasmalemma and tonoplast. Plant, Cell Environ. 7: 407-413

MACLENNAN, D.H., BRANDL, C.J., KORCZAK, B., GREEN, N.M. (1985) Amino-acid sequence of a Ca^{2+}+Mg^{2+}-dependent ATPase from rabbit muscle sarcoplasmic reticulum, deduced from its complementary DNA sequence. Nature 316: 696-700

MALATIALY, L., GREPPIN, H., PENEL, C. (1988) Ca^{2+} uptake by tonoplast and plasma membrane vesicles from spinach leaves. FEBS Lett. 233: 196-200

MANDALA, S., TAIZ, L. (1985) Solubilisation and partial purification of the tonoplast H^+-ATPase of corn coleoptiles. Plant Physiol. 78: 327-333

MANDALA, S., TAIZ, L. (1986) Characterisation of the subunit structure of the maize tonoplast ATPase. Immunological and inhibitor binding studies. J. Biol. Chem. 261: 12850-12855

MANOLSON, M.F., REA, P.A., POOLE, R.J. (1985) Identification of 3-0-(4-benzoyl)benzoyladenosine 5'-triphosphate and N,N'-dicyclohexyl-carbodiimide-binding subunits of a higher plant H^+-translocating ATPase. J. Biol. Chem. 260: 12273-12279

MARMÉ, D. (1982) The role of calcium in signal transduction of higher plants.In: Plant Growth Substances, 1982, pp 419-426 (WARING, P.F., Ed.) Academic Press, London, New York.

MARMÉ, D. (1985) The role of calcium in the cellular regulation of plant metabolism. Physiol. Veg. 23: 945-953

MARMÉ, D. (1986) The role of calcium in the regulation of plant metabolism. In: Molecular and cellular aspects of calcium in plant development, pp1-8 (TREWAVAS, A.J., Ed.) Nato ASI Series vol. 104. Plenum Press, New York, London.

MARMÉ, D., DIETER, P. (1983) The role of calcium and calmodulin in plants.In: Calcium and cell function, pp 263-311 (CHEUNG, W.Y., Ed.) Academic Press, London, New York.

MARTINY-BARON, G., SCHERER, G.F.E. (1988) A plant protein kinase and plant microsomal H^+ transport are stimulated by the ether phospholipid platelet-activating factor. Plant Cell Rep. 7: 579-582

McMANUS, M.T., McKEATING, J., SECHER, D.S., OSBORNE, D.J., ASHFORD, D., DWEK, R.A., RADEMACHER, T.W. (1988) Identification of a monoclonal antibody to abscission tissue that recognises xylose/fucose-containing N-linked oligosaccharides from higher plants. Planta 175: 506-512

McMURRAY, W.C., IRVINE, R.F. (1988) Phosphatidylinositol4,5-bisphosphate phosphodiesterase in higher plants. Biochem. J. 249: 877-881

MELIN, P-M., SOMMARIN, M., SANDELIUS, A.S., JERGI, B. (1987) Identification of Ca^{2+}-stimulated polyphosphoinositide phospholipase C in isolated plant plasma

membranes. FEBS Lett. 223: 87-91

MICHELL, B. (1986) Inositol phosphates: profusion and confusion. Nature 319: 176-177

MOHNEN, D., SHINSHI, H., FELIX, G., MEINS, F.Jr. (1985) Hormonal regulation of beta-1,3-glucanase messenger-RNA levels in cultured tobacco tissues. EMBO J. 4: 1631-1635

MOLL, C., JONES, R.L. (1981) Calcium and gibberellin-induced elongation of lettuce hypoctyl sections. Planta 152: 450-456

MORRÉ, D.J., GRIPSHOVER, B., MONROE, A., MORRÉ, J.T. (1984) Phosphatidylinositol turnover in isolated soybean membranes stimulated by the synthetic growth hormone 2,4-dichlorophenoxyacetic acid. J. Biol. Chem. 254: 15364-15368

MORRÉ, D.J., PFAFFMAN, H., DROBES, B., WILKINSON, F.E., HARTMAN, E. (1989) Diacylglycerol levels unchanged during auxin-stimulated growth of excised hypocotyl segments of soybean. Plant Physiol. 90: 275-279

MORRISEY, J.H. (1981) Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Analytical Biochem. 117: 307-310

MORSE, M.J., COTÉ, G.G., CRAIN, R.C., SATTLER, R.L. (1988) Light modulated phosphatidylinositol turnover in *Samanea saman* Plant Physiol. 86S: 93

MORSE, M.J., CRAIN, R.C., COTÉ, G.G., SATTER, R.L. (1989b) Light-stimulated inostol phospholipid turnover in *Samanea saman* pulvini. Plant Physiol. 89: 724-727

MORSE, M.J., CRAIN, R.C., SATTER, R.L. (1986) Phosphatidylinositol turnover in *Samanea* pulvini: a mechanism of phototransduction. Plant Physiol. 80S: 92

MORSE, M.J., CRAIN, R.C., SATTER, R.L. (1987a) Phosphatidylinositol cycle metabolites in *Samanea saman* pulvini Plant Physiol. 83: 640-644

MORSE, M.J., CRAIN, R.C., SATTER, R.L. (1987b) Light stimulated inositol phospholipid turnover in *Samanea saman.*Proc. Natl. Acad. Sci. USA 84: 7075-7078

MORSE, M.J., SATTER, R.L., CRAIN, R.C., COTÉ, G.G. (1989a) Signal transduction and phosphatidylinositol turnover in plants. Physiol. Plant. 76: 118-121

MUALLEM, S., KARLISH, S.J.D. (1980) Regulatory interaction between calmodulin and ATP on the red cell Ca^{2+} pump. Biochim. Biophys. Acta. 597: 631-636

MUTO, S., MIYACHI, S. (1977) Properties of a protein activator of NAD kinase from plants. Plant Physiol. 59: 55-60

NAGAHASHI, G., LEONARD, R.T., THOMPSON, W.W. (1978) Purification of plasma membranes from roots of barley. Specificity of the phosphotungstic acid-chromic acid stain. Plant Physiol. 61: 993-999

NEWTON, R.P., KINGSTON, E.E., EVANS, D.E., YOUNIS, L.Y., BROWN, E.G. (1984) Occurrence of guanosine 3':5'-cyclic monophosphate (cyclic GMP) and associated enzyme systems in *Phaseolus vulgaris.*Phytochemistry 13: 1367-1372

NEYSES, L., REINLIB, L., CARAFOLI, E. (1985) Phosphorylation of the calcium-pumping ATPase of heart sarcolemma and erythrocyte plasma membrane by the cyclic AMP-dependent protein kinase. J. Biol. Chem. 260: 10283-10287

NIGGLI, V., ADUNYAH, E.S., CARAFOLI, E. (1981b) Acidic phospholipids, unsaturated fatty acids and limited proteolysis mimic the effect of calmodulin on the purified erythrocyte Ca^{2+}-ATPase. J. Biol. Chem. 256: 8588-8592

NIGGLI, V., ADUNYAH, E.S., PENNISTON, J.T., CARAFOLI, E. (1981a) Purified (Ca^{2+}-Mg^{2+}) ATPase of the erythrocyte membrane: reconstitution and effect of calmodulin and phospholipids. J. Biol. Chem. 256: 395-401

NIGGLI, V., PENNISTON, J.T., CARAFOLI, E. (1979b) Purification of the

(Ca^{2+}+Mg^{2+})-ATPase from human erythrocyte membranes using a calmodulin-affinity column. J. Biol. Chem. 254: 9955-9958

NIGGLI, V., RONNER, P., CARAFOLI, E., PENNISTON, J.T. (1979a) Effect of calmodulin on the (Ca^{2+}+Mg^{2+}) ATPase partially purified from erythrocyte membranes. Arch. Biochem. Biophys. 198: 124-130

NIGGLI, V., SIGEL, E., CARAFOLI, E. (1982) The purified Ca^{2+}-pump of human erythrocyte membranes catalyses an electroneutral Ca^{2+}-H^{+} exchange in reconstituted liposomal systems. J. Biol. Chem. 257: 2350-2356

NIGGLI, V., ZURINI, M., CARAFOLI, E. (1987) Purification, reconstitution and molecular characterisation of the Ca^{2+} pump of plasma membranes. Meth. Enzymol 139: 791-808

NISHIZUKA, Y. (1986) Studies and perspectives of protein kinase C.Science 233: 305-312

NORMAN, P.M., WINGATE, M.S., FITTER, M.S., LAMB, C.J. (1986) Monoclonal antibodies to plant plasma-membrane antigens. Planta 167: 452-459

OLÁH, Z., BERCZI, A., ERDEI, L. (1983) Benzylaminopurine-induced coupling between calmodulin and Ca-ATPase in wheat root microsomal membranes. FEBS Lett. 154: 395-399

OLÁH, Z., BÖGRE, L., LEHEL, C., FARAGO, A., SEPRODI, J., DUDITS, D. (1989) The phosphorylation site of Ca^{2+}-dependent protein kinase from alfalfa. Plant Molec. Biol. 12: 453-461

OUCHTERLONY, Ö. (1967) Immunodiffusion and immunonoelectrophoresis. In: Handbook of experimental immunology, pp29-37 (WEIR, D.M., Ed.) Blackwell Scientific Publications, Oxford.

OWEN, J.H. (1988) Role of abscisic acid in a Ca^{2+} second messenger system. Physiol. Plant. 72: 637-641

OWEN, J.H., HETHERINGTON, A.M., WELLBURN, A.R. (1987) Calcium, calmodulin and the control of respiration in protoplasts isolated from menstematic tissues by abscisic acid. J. Exp. Bot. 38: 1356-1361

PALIYATH, G., POOVAIAH, B.W. (1984) Calmodulin inhibitor in senescing apples and its physiological and pharmacological significance. Proc. Natl. Acad. Sci. USA 81: 2065-2069

PALIYATH, G., POOVAIAH, B.W. (1988) Promotion of β-glucan synthase activity in corn microsomal membranes by calcium and protein phosphorylation.Plant Cell Physiol. 29: 67-73

PALIYATH, G., THOMPSON J.E. (1988) Senescence-related changes in ATP-dependent uptake of calcium into microsomal vesicles from carnation petals. Plant Physiol. 88: 295-302

PALMGREN, M.G., SOMMARIN, M. (1989) Lysophosphatidylcholine stimulates ATP-dependent proton accumulation in isolated oat root plasma membrane vesicles. Plant Physiol. 90: 1009-1014

PASTAN, I. (1972) Cyclic AMP. Sci. Am. 227: 97-105

PEDERSON, P.L., CARAFOLI, E. (1987a) Ion motive ATPases: I. Ubiquity, properties and significance to cell function. Trends. Biochem. Sci. 12: 146-150

PEDERSON, P.PL., CARAFOLI, E. (1987b) Ion motive ATPases: II. Energy coupling and work output. Trends Biochem. Sci. 12: 186-189

PENNISTON, J.T. (1983) Plasma membrane Ca^{2+}-ATPases as active Ca^{2+} pumps. In: Calcium and cell function, vol 4, pp 99-149 (CHEUNG, W.Y., Ed.) Academic Press,

London, New York.

PENNISTON, J.T., FILOTEO, A.G., McDONOUGH, C.S., CARAFOLI, E. (1988) Purification, reconstitution, and regulation of plasma membrane Ca^{2+} pumps. Methods Enzymol. 157: 340-351.

PENNISTON, J.T., GRAF, E., NIGGLI, V., VERMA, A.K., CARAFOLI, E. (1980) The plasma membrane calcium ATPase. In: Calcium binding proteins: structure and function, pp 89-103 (SIGEL, F.L., CARAFOLI, E., KRETSINGER, R.H., MACLENNAN, D.H., WASSERMAN, R.H., Eds.) Elsevier, Amsterdam, Oxford, New York, Tokyo.

PETERSON, S.W., RONNER, P., CARAFOLI, E. (1978) Partial purification and reconstitution of the (Ca^{2+}+Mg^{2+})-ATPase of erythrocyte membranes.Arch. Biochem. Biophys. 186: 202-210

POLYA, G.M., DAVIES, J.R. (1982) Resolution of a calcium-calmodulin activated protein kinase from wheat germ.FEBS Lett. 150: 167-171

POLYA, G.M., DAVIES, J.R., MICUCCI, V. (1983) Properties of a calmodulin-activated Ca^{2+}-activated protein kinase from wheat germ. Biochim. Biophys. Acta. 761: 1-12

POLYA, G.M., MICUCCI, V. (1984) Partial purification and characterisation of a second calmodulin activated calcium-dependent protein kinase from wheat germ. Biochim. Biophys. Acta. 785: 68-74

POLYA, G.M., KLUCIS, E., HARITOU, M. (1987) Resolution and characterisation of two soluble calcium-dependent protein kinases from silver beet leaves. Biochim. Biophys. Acta. 931: 68-77

POOVAIAH, B.W., LEOPOLD, A.C., (1973) Inhibition of abscission by calcium. Plant Physiol. 51: 848-851

POOVAIAH, B.W., LEOPOLD, A.C. (1976) Effects of inorganic solutes on the binding of auxin. Plant Physiol. 58: 783-785

POOVAIAH, B.W., McFADDEN, J.J., REDDY, A.S.N. (1987b) The role of calcium ions in gravity signal perception and transduction. Physiol. Plant. 71: 401-407

POOVAIAH, B.W., REDDY, A.S.N. (1987) Calcium messenger system in plants. CRC Crit. Rev. Plant Sci. 6: 47-103

POOVAIAH, B.W., REDDY, A.S.N., McFADDEN, J.J. (1987a) Ca^{2+} messenger system: role of protein phosphorylation and inositol bisphospholipids. Physiol Plant 69: 569-573

POST, R.L., SEN, A.K. (1967) [^{33}P]-labelling of a (Na^{+}-K^{+})-ATPase intermediate Methods Enzymol. 10: 762-768

POST, R.L., SEN, A.K., ROSENTHAL, A.S. (1965) A phosphorylated intermediate in adenosine triphosphatase dependent sodium and potassium transport across kidney membranes. J. Biol. Chem. 240: 1437-1445

PUTNAM-EVANS, C., HARMON, A.C., PALEVITZ, B.A., FECHHEIMER, M., CORMIER, M.J. (1989) Calcium-dependent protein kinase is localised with F-actin in plant cells. Cell Motil. Cytoskeleton. 12: 12-22

RACKER, E. (1972) Reconstitution of a calcium pump with phospholipids and a purified Ca^{++} adenosine triphosphatase from sacoplasmic reticulum. J. Biol. Chem. 247: 8198-8200

RACKER, E., VIOLAND, B., O'NEAL, S., ALFONZO, M., TELFORD, J. (1979) Reconstitution; a way of biochemical research some new approaches to membrane bound enzymes. Arch. Biochem. Biophys. 198: 470-477

RAGHOTHAMA, K.G., REDDY, A.S.N., FRIEDMANN, M., POOVAIAH, B.W.

(1987) Ca^{2+}-regulated *in vivo* protein phosphorylation in *Zea mays* L. root tips. Plant Physiol. 83: 1008-1013

RANDAL, S.K., SZE, H. (1986) Properties of the partially purified tonoplast H^+-pumping ATPase from oat roots. J. Biol. Chem. 261: 1364-1371

RANJEVA, R., GRAZIANA, A., RANTY, B., CAVALIE, G., BOUDET, A.M. (1984) Phosphorylation of proteins in plants: a step in the integration of extra and intracellular stimuli. Physiol. Veg. 22: 365-371

RANJEVA, R., GRAZIANA, A., DILLENSCHNEIDER, M., CHARPENTEAU, M., BOUDET, A.M. (1986) A novel plant calciprotein as a transient subunit of enzymes. In: Molecular and cellular aspects of calcium in plant development, pp 41-48 (TREWAVAS, A.J., Ed.) NATO ASI Series, vol 104, Plenum Press, New York, London.

RANJEVA, R., REFENO, G., BOUDET, A.M., MARMÉ, D. (1983) Plant quinate: NAD oxidoreductase is activated by Ca^{2+}-calmodulin dependent phosphorylation. Proc. Natl. Acad. Sci. USA 80: 5222-5224

RASI-CALDOGNO, F., DE MICHELIS, M.I., PUGLIARELLO, M.C. (1982) Active transport of Ca^{2+} in membrane vesicles from pea: evidence for a H^+/Ca^{2+} antiport Biochim. Biophys. Acta. 693: 287-295

RASI-CALDOGNO, F., PUGLIARELLO, M.C., DE MICHELIS, M.I. (1987) The Ca^{2+}-transport ATPase of plant plasma membrane catalyses a nH^+/Ca^{2+} exchange. Plant Physiol. 83: 994-1000

RASMUSSEN, H., BARRET, P.Q. (1984) Calcium messenger system: an integrated view. Physiol. Rev. 64: 938-984

RAUSCH, T., BUTCHER, D.N., TAIZ, L. (1987) Active glucose transport and proton pumping in tonoplast membrane of *Zea mays* L. coleoptiles are inhibited by anti-H^+ ATPase antibodies. Plant Physiol. 85: 996-999

REA, P.A., SANDERS, D. (1987) Tonoplast energisation: two H^+ pumps, one membrane. Physiol. Plant. 71: 131-141

REDDY, A.S.N., POOVAIAH, B.W. (1987) Inositol 1,4,5-trisphosphate induced calcium release from corn coleoptile microsomes. J. Biochem. 101: 569-573

REGA, A.F., GARRAHAN, P.J. (1975) Calcium ion-dependent phosphorylation of human erythrocyte membranes. J. Memb. Biol. 22: 313-327

REGA, A.F., GARRAHAN, P.J. (1980) Effects of calmodulin on the phosphoenzyme of the Ca^{2+}-ATPase of human red cell membranes. Biochim. Biophys. Acta. 596: 487-489

REGA, A.F., GARRAHAN, P.J. (1986) The Ca^{2+} pump of plasma membranes. CRC Press, Boca Raton, Florida, USA.

REGA, A.F., RICHARDS, D.E., GARRAHAN, P.J. (1973) Calcium ion-dependent p-nitrophenylphosphate phosphatase activity and calcium ion-dependent adenosine triphosphatase activity from human erythrocyte membranes. Biochem. J. 136: 185-194

REISS, H.-D., NOβILING, R. (1986) Quin-2 fluorescence in lily pollen tubes: distribution of free cytoplasmic calcium. Protoplasma 131: 244-246

REISS, H.-D., HERTH, W., SCHNEPE, E. (1986) Calcium and polarity in tip growing plant cells. In: Molecular and cellular aspects of calcium in plant development, pp 211-218 (TREWAVAS, A.J., Ed.) NATO ASI Series vol 104, Plenum Press, New York, London.

REUTER, H. (1983) Calcium channel modification by neurotransmitters, enzymes and drugs. Nature 301: 569-574

REUTER, H. (1985) A variety of calcium channels. Nature 316: 391

RINCON, M., BOSS, W. (1986) IP_3 stimulates Ca^{2+} efflux from fusogenic carrot protoplasts. Plant Physiol. 80S: 89

RINCON, M., BOSS, W.F. (1987) *myo*-inositol trisphosphate mobilises calcium from fusogenic carrot (*Daucus carota* L.) protoplasts. Plant Physiol. 83: 395-398

RINCON, M., CHEN, Q., BOSS, W.F. (1989) Characterisation of inositol phosphates in carrot (*Daucus carota* L.) cells.Plant Physiol. 89: 126-132

ROBERTS, D.M., LUKAS, T.J., WATTERSON, D.M. (1986) Structure, function and mechanism of action of calmodulin. CRC Crit. Rev. Plant Sci. 4: 311-339

ROBINSON, C., LARSSON, C., BUCKHOUT, T.J. (1988) Identification of a calmodulin-stimulated (Ca^{2+}+Mg^{2+})-ATPase in a plasma membrane fraction isolated from maize (*Zea mays*) leaves. Physiol. Plant. 72: 177-184

ROUX, S.J., SERLIN, B.S. (1987) Cellular mechanisms controlling light-stimulated gravitropism: role of calcium.CRC Crit. Rev. Pl. Sci. 5: 205-236

RUDNICK, G. (1986) ATP-driven H^+ pumping into intracellular organelles. Annu. Rev. Physiol. 48: 403-413

RYAN, C.A., WALKER-SIMMONS, M. (1981) Plant Proteinases. In: The biochemistry of plants, vol 6, pp 321-350 (MARCUS, A., Ed.) Academic Press, London, New York.

SANDELIUS, A.S., SOMMARIN, M. (1986) Phosphorylation of phosphatidylinositols in isolated plant membranes. FEBS Lett. 201: 282-286

SARKADI, B. (1980) Active calcium transport in human red cells.Biochim. Biophys. Acta. 604: 159-190

SATTER, R.L., GALSTON, A.W. (1981) Mechanisms of control of leaf movements. Annu. Rev. Plant Physiol. 32: 83-110

SCALLA, R., AMORY, A., RIGAUD, J., GOFFEAU, A. (1983) Phosphorylated intermediate of a transport ATPase and activity of protein kinase in membranes from corn roots.Eur. J. Biochem. 132: 525-530

SCHÄFER, A., BYGRAVE, F., MATENAUER, S., MARMÉ, D. (1985) Identification of a calcium- and phospholipid-dependent protein kinase in plant tissue. FEBS Lett. 187: 25-28

SCHALLER, G.E., SUSSMAN, M.R. (1988) Phosphorylation of the plasma-membrane H^+-ATPase of oat roots by a calcium-stimulated protein kinase.Planta 173: 509-518

SCHATZMAN, H.J. (1982) The plasma membrane calcium pump of erythrocytes and other animal cells.In: Membrane transport of calcium, pp 41-108 (CARAFOLI, E., Ed.) Academic Press, London, New York.

SCHERER, G.F.E. (1985) 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine (platelet activating factor) stimulates plant H^+ transport *in vitro* and growth. Biochem. Biophys. Res. Commun. 133: 1160-1167

SCHERER, G.F.E., MORRÉ, J.D. (1978) *In vitro* stimulation by 2,4-dichloro phenoxyacetic acid of an ATPase and inhibition of phosphatidate phosphatase of plant membranes. Biochem. Biophys. Res. Commun. 84: 238-247

SCHERER, G.F.E., STOFFEL, B. (1987) A plant phospholipid and platelet activating factor stimulate H^+ transport in isolated membrane vesicles. Planta 172: 127-130

SCHERER, G.F.E., MARTINY-BARON, G., STOFFEL, B. (1988) A new set of regulatory molecules in plants: a plant phospholipid similar to platelet-activating factor stimulates protein kinase and proton-translocating ATPase in membrane vesicles. Planta 175: 241-253

SCHNEIDER, H.A.W., LEIDGENS, W. (1981) An evolutionary tree based on monoclonal antibody-recognised surface features of plastid enzyme (5-aminolevulinate dehydratase). Z. Naturforsch 36c: 44-50

SCHUMAKER, K.S., SZE, H. (1985) A Ca^{2+}/H^{+} antiport system driven by the proton electrochemical gradient of a tonoplast H^{+}-ATPase from oat roots. Plant Physiol. 79: 1111-1117

SCHUMAKER, K.S., SZE, H. (1986) Calcium transport into the vacuole of oat roots. Characterisation of H^{+}/Ca^{2+} exchange activity. J. Biol. Chem. 261: 12172-12178

SEALS, J.R., McDONALD, J.M., BRUNS, D., JARETT, L. (1978) A sensitive and precise isotopic assay of ATPase activity. Anal. Biochem. 90: 785-795

SEKAR, M.C., HOKIN, L.E. (1986) The role of phosphoinositides in signal transduction. J. Memb. Biol. 89: 193-210

SERRANO, R. (1988) Structure and function of protein translocating ATPase in plasma membranes of plants and fungi.Biochim. Biophys. Acta 947: 1-28

SERRANO, R. (1989) Structure and function of plasma membrane ATPase. Annu. Rev. Plant Physiol. Plant Mol. Biol. 40: 61-94

SHANNON, J.D., WALLACE, W. (1979) Isolation and characterisation of peptide hydrolases from the maize root. Eur. J. Biochem. 102: 399-408

SHARMA, R.K., TAYLOR, W.A., WANG, J.H. (1983) Use of calmodulin affinity chromatography for purification of specific calmodulin-dependent enzymes. Methods Enzymol. 102: 210-219.

SIGEL, E., AFFOLTER, H. (1987) Preparation and utilization of an ion specific calcium minielectrode. Methods Enzymol. 141: 25-36

SIMON, W., CARAFOLI, E. (1979) Design, properties and applications of neutral

ionophores. Methods Enzymol. 56: 439-448

SMALLWOOD, J.I., GUGI, B., RASMUSSEN, H. (1988) Regulation of erythrocyte calcium pump activity by protein kinase C. J. Biol. Chem. 263: 2195-2202

SMITH, T.J., DAVIS, F.B., DAVIS, P.J. (1989) Retinoic acid is a modulator of thyroid hormone activation of calcium ATPase in the human erythrocyte membrane. J. Biol. Chem. 264: 687-689

de ST. GROTH, F., SCHEIDEGGER, D. (1980) Production of monoclonal antibodies: strategy and tactics. J. Immunol. Methods 35: 1-21

STIEGER, J., LUTERBACHER, S. (1981) Some properties of the purified (Ca^{2+}+Mg^{2+})-ATPase from human red cell membranes. Biochim. Biophys. Acta 641: 270-275

STOSIC, V., PENEL, C., MARMÉ, D., GREPPIN, H. (1983) Distribution of calmodulin-stimulated Ca^{2+}-transport into membrane vesicles from green spinach leaves. Plant Physiol. 72: 1136-1138

STRYER, L., BOURNE, H.R. (1986) G proteins: a family of signal transducers. Annu. Rev. Cell Biol. 2: 391-419

SUTHERLAND, E.W. (1972) Studies on the mechanism of hormone action. Science 177: 401-408

SZASZ, I., HASITZ, M., SARKADI, B., GARDOS, G. (1978) Phosphorylation of the Ca^{2+} pump intermediate in intact red cells, isolated membranes and inside-out vesicles. Molec. Cellular Biochem. 22: 147-152

SZE, H. (1985) H^{+}-translocating ATPases: advances using membrane vesicles, Annu. Rev. Plant Physiol. 36: 175-208

THEOLOGIS, A. (1986) Rapid gene regulation by auxin. Annu. Rev. Plant Physiol.

37: 407-438

THOMAS, M.V. (1982) Techniques in calcium research.Academic Press, London, New York.

THOMAS, M.V. (1986) The definition and measurement of intracellular free calcium. In: Molecular and cellular aspects of calcium in plant development, pp 141-148 (TREWAVAS, A.J., Ed.) NATO ASI Series vol 104, Plenum Press, New York, London.

TOBIN, E.M., SILVERTHORNE, J. (1985) Light regulation of gene expression in higher plants. Annu. Rev. Plant Physiol. 36: 569-593

TODA, H., YAZAWA, M.M, SAKIYAMA, F., YAGI, K. (1985) Amino acid sequence of calmodulin from wheat germ. J. Biochem. 98: 833-842

TOKUMURA, A., MOSTAFA, M.H., NELSON, D.R., HANAHAN, D.J. (1985) Stimulation of calcium-magnesium ATPase activity in human erythrocyte membranes by synthetic lysophosphatidic acids and lysophosphatidyl cholines: effects of chain length and degree of unsaturation of the fatty acid groups. Biochim. Biophys. Acta. 812: 568-574

TORIYAMA, H., JAFFE, M.J. (1972) Migration of calcium and its role in the regulation of seismonasty in the motor cell of *Mimosa pudica* L. Plant Physiol. 49: 72-81

TOWBIN, H., STAEHELIN, T., GORDON, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76: 4350-4354

TREWAVAS, A.J. (1976) Post-translational modification of proteins by phosphorylation. Annu. Rev. Plant Physiol. 27: 349-374

TREWAVAS, A.J. (ed) (1986) Molecular and cellular aspects of calcium in plant development. Nato ASI Series vol 104. Plenum Press, New York, London.

Van ELDIK, L.J., PIPERNO, G., WATTERSON, D.M. (1980) Similarities and dissimilarities between calmodulin and a *Chlamydomonas* flagellar protein. Proc. Natl. Acad. Sci. USA 77: 4779-4783

Van der LINDE, P.C.G., BOUMAN, H., MENNES, A.M., LIBBENGE, K.R. (1984) A soluble auxin-binding protein from cultured tobacco tissues stimulates RNA synthesis *in vitro*. Planta 160: 102-108.

VAN DER WOUDE, W.J., LEMBI, C.A., MORRÉ, D.J., KINDINGER, J.I., ORDIN, L. (1974) β-glucan synthetases of plasma membrane and Golgi apparatus from onion stem. Plant Physiol. 54: 333-340

VARA, F., SERRANO, R. (1982) Partial purification and properties of the H^+-translocating ATPase of plant plasma membranes. J. Biol. Chem 257: 12826-12830

VARA, F., SERRANO, R. (1983) Phosphorylated intermediate of the ATPase of plant plasma membranes. J. Biol. Chem. 258: 5334-5336

VELUTHAMBI, K., POOVAIAH, B.W. (1984a) Calcium promoted phosphorylation in plants. Science 223: 167-169

VELUTHAMBI, K., POOVAIAH, B.W. (1984b) Calcium and calmodulin-regulated phosphorylation of soluble and membrane proteins from corn coleoptiles. Plant Physiol. 76: 359-365

VERMA, A.K., PENNISTON, J.T. (1981) A high affinity Ca^{2+}-stimulated and Mg^{2+}-dependent ATPase in rat corpus luteum plasma membrane fractions. J. Biol. Chem. 256: 1269-1275

VERMA, A., GORSKI, J., PENNISTON, J. (1982) Antibodies directed toward human erythrocyte Ca^{2+} ATPase: effect on enzyme function and immunoreactivity of Ca-ATPases from other sources. Arch. Biochem. Biophys. 215: 345-354

VERMA, A.K., PENNISTON, J.T., MUALLEM, S., LEW, V. (1984) Effects of affinity purified antibodies on the Ca^{2+} pumping ATPase of erythrocyte membranes. J. Bioenerg. and Biomemb. 16: 365-378

VERMA, A.K., FILOTEO, A.G., STANFORD, D.R., WIEBEN, E.D., PENNISTON, J.T., STREHLER, E.E., FISCHER, R., HEIM, R., VOGEL, G., MATHEWS, S., STREHLER-PAGE, M.A., JAMES, P., VORHERR, T., KREBS, J., CARAFOLI, E. (1988) Complete primary structure of a human plasma membrane Ca^{2+} pump. J. Biol. Chem. 263: 14152-14159

VILLANUEVA, M.A., METCALF, T.N., WANG, J.L. (1986) Monoclonal antibodies directed against protoplasts of soybean cells. Generation of hybridomas and characterisation of a monoclonal antibody reactive with the cell surface. Planta 168: 503-511

WANG, T.L. (ed.) (1986) Immunology in plant science. Society for Experimental Biology Seminar Series, vol 29. Cambridge University Press, Cambridge.

WANG, K.K.W., VILLALOBO, A., ROUFOGALIS, B.D. (1988) Activation of the calcium ATPase of human erythrocyte membrane by an endogenous calcium dependent neutral protease. Arch. Biochem. Biophys. 260: 696-704

WARBURG, O., CHRISTIAN, W. (1941) Isolierung und kristallisation des Gärungsferments enolase. Biochemische Zeitschrift 310: 384-421

WEBER, K., OSBORNE, M. (1969) The reliability of molecular weight determinations by dodecyl sulfate polyacrylamide gel electrophoresis. J. Biol. Chem. 244: 4406-4412

WHEELER, J.J., BOSS, W.F. (1987) Polyphosphoinositides are present in plasma membranes isolated from fusogenic carrot cells. Plant Physiol. 85: 389-392

WILLIAMSON, R.E. (1981) Free Ca^{2+} concentration in the cytoplasm: a regulator of plant cell function. What's New Plant Physiol. 12: 45-48

WOLF, H.U., DIECKVOSS, G., LICHTNER, R. (1976) Partial purification of soluble high-affinity Ca^{2+}-ATPase of human erythrocyte membranes. Expeerientia 32: 776

WOLF, H.U., DIECKVOSS, G., LICHTNER, R. (1977) Purification and properties of high-affinity Ca^{2+}-ATPase of human erythrocyte membranes. Acta. Biol. Med. Ger. 36: 847-858

WYCOFF, K.L., JELLISON, J., AYERS, A.R. (1987) Monoclonal antibodies to glycoprotein antigens of a fungal plant pathogen *Phytophthora megasperma* f.sp. *glycinea*. Plant Physiol. 85: 508-515

YAMAMOTO, K.R. (1985) Steroid receptor regulated transcription of specific genes and gene networks. Annu. Rev. Genet. 19: 209-252

ZBELL, B., WALTER, C. (1988) Signal transduction of auxin isolated plant cell membranes: indications for a rapid polyphosphoinositide response stimulated by indole acetic acid. J. Plant Physiol. 133: 353-360

ZOCCHI, G. (1985) Phosphorylation-dephosphorylation of membrane proteins controls the microsomal H^{+}-ATPase activity of corn roots. Plant Sci. 40: 153-159

ZOCCHI, G. (1988) Separation of membrane vesicles from maize roots having different calcium transport activities. Plant Sci. Lett. 54: 103-107

ZOCCHI, G., HANSON, J.B. (1983) Calcium transport and ATPase activity in a microsomal vesicle fraction from corn roots. Plant Cell Environ. 6: 203-209

ZOCCHI, G., ROGERS, S.A., HANSON, J.B. (1983) Inhibition of proton pumping in corn roots is associated with increased phosphorylation of membrane proteins. Plant Sci. Lett. 31: 215-221

ZURINI, M., KREBS, J., PENNISTON, J.T., CARAFOLI, E. (1984) Controlled proteolysis of the purified Ca-ATPase of the erythrocyte membrane: a correlation between

the structure and function of the enzyme. J. Biol. Chem. 259: 618-627

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THE CALMODULIN-STIMULATED ATPase OF MAIZE COLEOPTILES FORMS A PHOSPHORYLATED INTERMEDIATE

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SUMMARY The calmodulin-stimulated ATPase of maize coleoptiles is a 140,000 M_r polypeptide. In the present study, formation of a phosphorylated intermediate by the enzyme is demonstrated. Phosphorylation is sensitive to chasing with unlabelled ATP and to hydroxylamine; lanthanum enhances its intensity while calmodulin enhances phosphorylation in the presence of lanthanum but not in its absence. Ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) inhibits phosphorylation of the purified enzyme, but microsome preparations give a band of phosphorylation of 153,000 M_r in its presence. This latter phosphorylated band was not abolished after a variety of permeabilising treatments in the presence of Triton X-100; phosphorylation of the enzyme was absent when sodium deoxycholate was used as the solubilising detergent. The identity of the 153,000 M_r band is discussed. © 1989 Academic Press, Inc.

Maize (Zea mays L.) coleoptile plasma membrane contains a calmodulin-stimulated calcium-pumping ATPase (1,2), which catalyzes an nH^+/Ca^{2+} exchange (3). This enzyme has previously been shown (4) to be a polypeptide which shows common antigenicity with the erythrocyte membrane calcium pump, and has a similar M_r (140,000). The higher plant enzyme is believed to be the major transporter responsible for the maintenance of low cytoplasmic free calcium concentrations in higher plant cells (5). It is activated in the presence of increased calcium concentrations by the calcium-dependent regulator protein calmodulin (1).

In order to investigate the properties of the maize calmodulin-stimulated ATPase further, we have studied both the formation of phosphorylated intermediates by the purified enzyme and by microsomes containing it. Phosphorylated intermediate formation is a characteristic of ion-pumping

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ATPases of the E_1-E_2 type. The intermediate is an aspartyl phosphate which is sensitive to treatment with hydroxylamine and is sensitive to basic conditions. Turnover of the intermediate is rapid (6), but is stabilised by the presence of lanthanum (7) and acidic conditions such that it may readily be demonstrated using ATP labelled in the γ position with ^{32}P followed by precipitation of the protein in acidic conditions, separation of the proteins using polyacrylamide gel electrophoresis at pH 6 and autoradiography.

MATERIAL AND METHODS

Tissue and enzyme purification:
Enzyme and microsomes were extracted from 4.5d dark-grown maize (cv. Golden Bantam) seedlings by the methods described previously (4). The proteins in the "washed" microsome pellet were solubilised with Triton X-100 (0.1mg/mg protein or 1mg/mg protein), or sodium deoxycholate (1mg/mg protein) as indicated in the text. Where appropiate, the microsomes were permeabilised by being subjected to freeze/thaw cycles, vortexed for 10 min, or sonicated for 3 min in bursts of 15 s with 15 s cooling intervals (Dawe soniprobe, fine tip).

Phosphorylated intermediate formation:
The formation of phosphorylated intermediates in the microsome fraction and in purified enzyme was carried out by the method used previously for the erythrocyte calcium pumping ATPase (8). To the assays, microsome sample (100-200µg protein) or purified enzyme (2-10µg protein) were added. The reaction was started by the addition of either 37KBq/assay [γ-^{32}P]-ATP (New England Nuclear, 111MBq/mmol) or 370KBq/assay [γ-^{32}P]-ATP plus 2µM unlabelled ATP (Sigma, vanadate free) as indicated in text. The assay was conducted in Eppendorf tubes, which were incubated for 15 s on ice before reactions were terminated by addition of trichloroacetic acid (TCA) to 5% (w/v) final concentration. Calcium chloride (50µM final concentration), ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA, 500µM final concentration), lanthanum chloride (50µM final concentration) and calmodulin (Sigma, bovine brain, 25,000 units/0.53mg solid) were included as indicated in text. Where appropriate, 50µl 50mM unlabelled ATP was added for 1 min before addition of TCA. Hydroxylamine treatment was accomplished by washing the precipitated protein in 50mM hydroxylamine in 100mM 2(N-morpholino)ethanesulphonic acid (MES) brought to pH 6.0 with KOH. Control treatment (washing in MES/KOH) was also carried out and did not diminish phosphorylation. The pelleted protein was washed once in water and resuspended for electrophoresis in sodium phosphate sample buffer (pH 6.0) and incubated on ice for 30 min prior to electrophoresis on 5% or 7.5% gels prepared according to the method of Weber and Osborne (9) modified to pH 6.0. Gels were dried without fixation using a Biorad 483 slab gel drier and exposed for 1 d (microsome preparations) or 14 d (purified enzyme) at -70°C with Kodak X-omat S autoradiography film in the presence of a Du Pont Cronex Lightning-Plus intensifier screen. Gels were stained using Coomassie blue as described in (4) or by the method of (10). Values for M_r were determined by comparison of autoradiographs with M_r standards (Sigma SDS-6H) run in adjacent gel lanes which were cut out and stained as described in (4). Values given are representative of five experiments.

RESULTS AND DISCUSSION

Phosphorylation in presence of calcium:

In the presence of calcium, microsomes and purified enzyme showed a 140,000 M_r band of phosphorylation after 15 s on ice both in the presence of 37KBq

[γ^{32}P]-ATP per assay (fig.1, lane 1; fig.2, lane 2) and in the presence of 370KBq [γ-^{32}P]-ATP per assay plus 2μM unlabelled ATP (data not shown). In both cases, the 140,000 M_r polypeptide was not phosphorylated after chasing with unlabelled ATP (fig.1, lane 2; fig.2, lane 6) or after washing with hydroxylamine (fig.1, lane 3; fig.2, lane 7), indicating that it was an acyl-phosphate of the type described for ion-translocating ATPases of the E_1-E_2 type. This finding is identical to that of Niggli et al. (8) for the erythrocyte calcium-pumping ATPase and is within ±5,000 of the M_r established for the phosphorylated intermediate of that enzyme (11).
Two other bands of phosphorylation were also present in the microsome extract, one at 44,000 M_r and one at 28,000 M_r. The former (absent from the purified enzyme preparation) was not diminished by hydroxylamine and may, therefore be assumed to be the result of protein kinase activity. The latter was abolished by hydroxylamine and may be the result of partial proteolysis of the enzyme after phosphorylation.

Effect of calmodulin and lanthanum:
When microsome extracts and purified enzyme were incubated with lanthanum, apparent phosphorylation was increased as compared with that in the presence of calcium (fig. 3, lanes 2 and 4; fig. 2, lanes 2 and 4). Lanthanum reduces turnover of phosphorylated intermediates and thereby stabilises the phosphorylated state, resulting in apparent enhancement of phosphorylation (6). No reproducible effect of calmodulin (25 units/assay) on phosphorylation in the absence of lanthanum could be detected either in the case of microsomes or purified enzyme (fig. 3, lanes 1 and 2; fig. 2, lanes 1 and 2, apparent increased band intensity in lane 1, fig. 2 being the result of slightly heavier loading of that lane and not being found in all replicate experiments). However, in the presence of lanthanum, slight band intensification in the presence of calmodulin was suggested, at least for purified enzyme (fig. 2, lanes 3 and 4). This intensification could be demonstrated reproducibly. These effects of calmodulin are likely to indicate that increased enzyme activity in the presence of calmodulin is the result of increased turnover of the phosphorylated state rather than an increase in total phosphorylation at any instant. In the presence of lanthanum, inhibition of dephosphorylation results in the increased phosphorylation in the presence of calmodulin being detected as an intensified band.

Effect of EGTA:
When microsomes solubilsed with 0.1mg Triton X-100/mg protein were incubated in the presence of 500μM EGTA, a band of phosphorylation at M_r 153,000 was apparent while that at M_r 140,000 disappeared (fig. 4, lanes 1 and 2). This

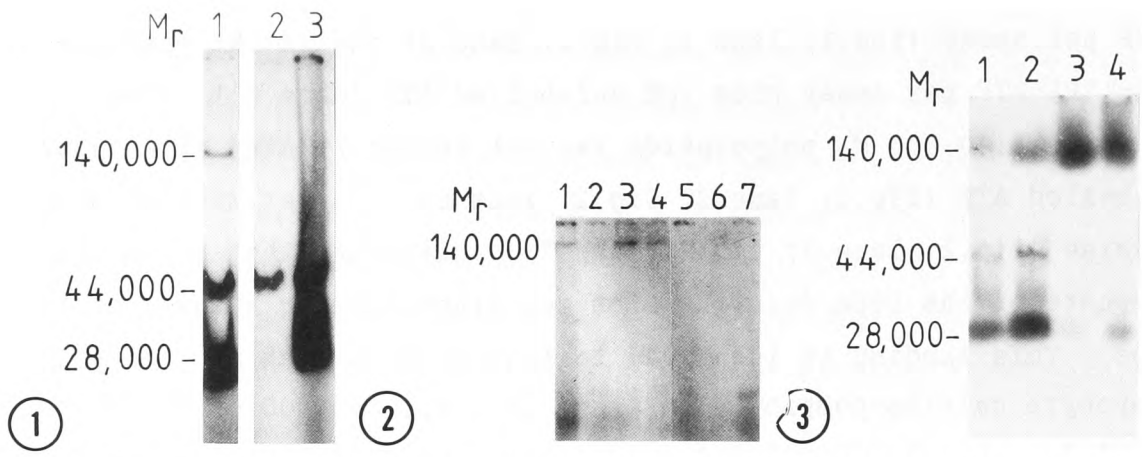


Figure 1. Phosphorylated intermediate formation by microsome fractions in the presence of calcium; autoradiograph of 5% pH 6.0 SDS polyacrylamide gel. Lane 1: membrane phosphorylated on ice for 15 s in the presence of 50μM added $CaCl_2$: lane 2: as lane 1 but 5mM unlabelled ATP added for 1 min after phosphorylation; lane 3: as lane 1 but pelleted membrane preparation washed with 50mM hydroxylamine in 100mM MES/KOH pH 6.0 prior to electrophoresis.

Figure 2. Phosphorylated intermediate formation by purified enzyme; autoradiograph of 7.5% pH 6.0 SDS polyacrylamide gel. Lane 1: enzyme incubated for 15 s on ice in the presence of 50μM added $CaCl_2$ and 25 units calmodulin; lane 2: as 1 but without calmodulin; lane 3: as 1 with 50μM $LaCl_3$ plus calmodulin; lane 4: as 3 without calmodulin; lane 5: as 1 but with 500μM EGTA and without calmodulin; lane 6: as lane 1 but 5mM unlabelled ATP added for 1 min after phosphorylation; lane 7: as lane 1 but pelleted protein washed with 50mM hydroxylamine in 100mM MES/KOH pH 6.0 prior to electrophoresis.

Figure 3. Effect of lanthanum and calmodulin on phosphorylated intermediate formation by microsomes. Autoradiograph of 5% pH 6.0 SDS polyacrylamide gel. Lane 1: Microsomes phosphorylated for 15 s on ice in the presence of 50μM added calcium plus 25 units calmodulin; lane 2: as 1, without calmodulin; lane 3: as lane 1 but with 50μM $LaCl_3$ plus calmodulin; lane 4: as 3, without calmodulin.

band was abolished by an ATP chase (fig. 4, lane 3), and was hydroxylamine sensitive (fig. 4, lane 4). To investigate whether persistence of phosphorylation in the presence of EGTA was due to the microsomes being insufficiently permeabilised to permit the chelation of calcium in the immediate environment of the enzyme by EGTA, a series of permeabilisation and solubilisation treatments were carried out. Tip sonicating or vortexing the microsomes solubilised with 0.1mg Triton X-100/mg protein did not diminish the phosphorylation of the 153,000 M_r polypeptide in the presence of EGTA (Fig. 5a, lanes 1-4). Freeze/thawing the microsomes solubilised with 0.1mg Triton X-100/mg protein two, three or four times did not diminish the phosphorylation at 153,000 M_r in the presence of EGTA (Fig. 5b, lanes 1-6). In the presence of higher Triton X-100 concentrations (1mg/mg protein) the phosphorylated band was still present with EGTA (Fig. 6, lanes and 2). The use of sodium deoxycholate to solubilise the membrane proteins results in the phosphorylation of the enzyme being abolished, whether in the presence of calcium or EGTA (Fig. 6, lanes 3 and 4).

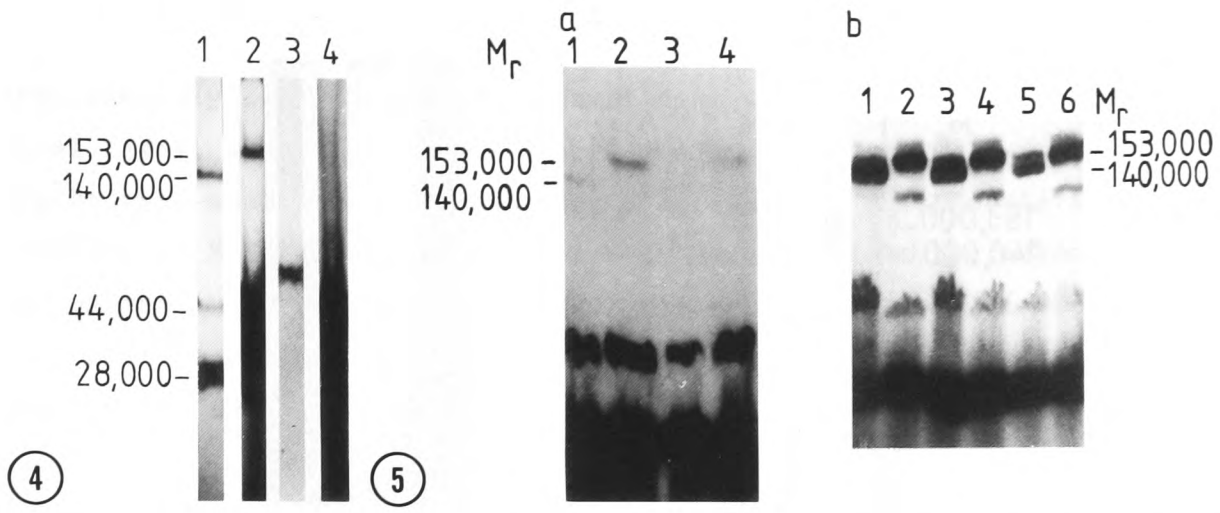
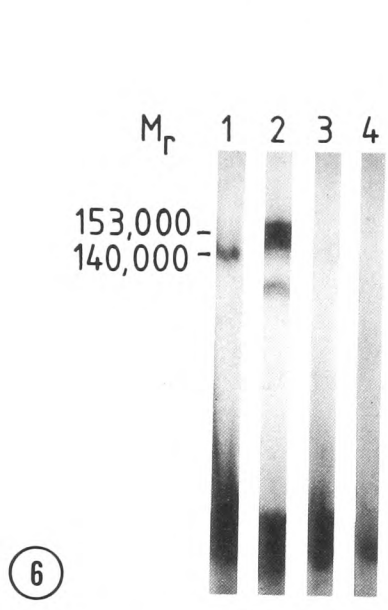


Figure 4. Effect of EGTA on microsome preparations. Autoradiograph of 5% pH 6.0 SDS polyacrylamide gel. Lane 1: microsomes phosphorylated in the presence of 50μM $LaCl_3$ for 15 s on ice; lane 2: as lane 1 but with 500μM EGTA in place of $LaCl_3$; lane 3: as lane 2 but 5mM unlabelled ATP added for 1 min after phosphorylation; lane 4: as lane 2, but pelleted membrane preparation washed with 50mM hydroxylamine in 100mM MES/KOH pH 6.0 prior to electrophoresis.

Figure 5. Formation of phosphorylated intermediates in microsomes, autoradiographs of 5% pH 6.0 SDS polyacrylamide gels. (a) After permeabilising treatments. Lane 1: microsomes phosphorylated after sonication with 0.1mg Triton X-100/mg protein in the presence of calcium; lane 2: as lane 1, but with 500μM EGTA in place of calcium; lane 3: as lane 1, but microsomes vortexed for 10 min; lane 4: as lane 3, but with 500μM EGTA. (b) After freeze/thaw treatments. Lane 1: microsomes phosphorylated after two freeze/thaw cycles in the presence of calcium; lane 2: as lane 1, but with 500μM added EGTA in place of calcium; lanes 3 and 4, as lanes 1 and 2 respectively, but microsomes phosphorylated after three freeze/thaw cycles; lanes 5 and 6: as lanes 1 and 2 respectively, but microsomes phosphorylated after four freeze/thaw cycles.

Phosphorylation of the purified enzyme preparation differed from that of the microsome preparation in that it showed calcium dependence. The band of phosphorylation at 140,000 M_r in the presence of calcium was not apparent in the presence of EGTA (fig. 2, lane 5). No band of phosphorylation at 153,000 M_r appeared and when polyacrylamide gels prepared in parallel were silver stained for protein (Fig. 7), no differences in M_r between Ca^{2+} treated and EGTA treated enzyme was apparent. (It should be noted that the presence of the lower M_r polypeptides visible in the purified enzyme preparation on pH 6.0 gels are likely to be the result of proteolysis of the purified enzyme; comparable gels under neutral conditions have been presented previously (4) and evidence for partial proteolysis is discussed therein). Phosphorylation of the erythrocyte calcium pumping ATPase is calcium sensitive (8), both in membrane preparations and in the purified enzyme and it thus appears that there may be differences between it and the plant enzyme in this respect. While it is possible that the 153,000 M_r phosphorylation is due to the presence of another ATPase in the membrane preparation which is inhibited in the presence of calcium, it is feasible that it is the calmodulin-stimulated



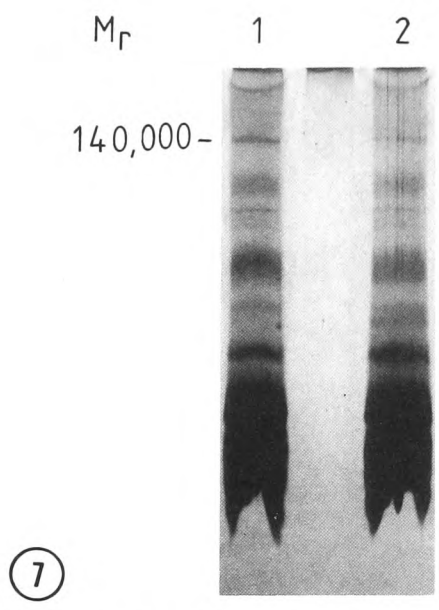


Figure 6. Phosphorylated intermediate formation by microsome preparations after solubilisation with increased detergent concentrations; autoradiograph of 5% pH 6.0 SDS polyacrylamide gel. Lane 1: membrane phosphorylated on ice for 15 s after solubilisation with 1mg Triton X-100/mg protein in the presence of 50μM added calcium, lane 2: as lane 1 but in the presence of 500μM EGTA in place of calcium; lanes 3 and 4: as lanes 1 and 2 respectively, but microsome proteins solubilised with 1mg sodium deoxycholate/mg protein.

Figure 7. Effect of EGTA on purified enzyme. Purified enzyme preparation was incubated with 500μM EGTA for 15 s on ice and precipitated with TCA. The pellet was then prepared for electrophoresis as described in 'Methods' and separated on 5% SDS polyacrylamide gels. The resulting gels were silver stained by the method of (10).

enzyme running at an anomalous M_r in the presence of EGTA. If this is the case, not only is a marked conformational change indicated (not noted in the purified erythrocyte enzyme or in the purified plant enzyme) but the membrane-associated enzyme also appears to phosphorylate in the presence of EGTA, even after extensive microsome-permeabilising treatments. As yet, an antibody which recognises the calmodulin-stimulated enzyme in microsome preparations is not available; therefore it has not been possible to locate the calmodulin-stimulated enzyme in microsomes in the presence of calcium or EGTA by any method other than by the formation of phosphorylated intermediates, and thereby to unequivocally identify the 153,000 M_r polypeptide as the calcium pump. As the purified enzyme behaves similarly to the erythrocyte enzyme in being calcium-sensitive, it appears that any differences lie in the inter-relationship between the enzyme and the membrane. Possibly the plant membrane retains sufficient strongly bound calcium to locally overcome the effect of the EGTA. Clearly, no entirely satisfactory explanation for the effect of EGTA on phosphorylation in membrane preparations will be possible until considerably more information on the membrane environment and its effects on the enzyme is obtained. It is interesting to note that Knauf et al. (12) detected a calcium-stimulated phosphoprotein in red blood cell ghosts that consisted of two components - a faint band of

approximately 142,000 M_r and a denser band of 150,000 M_r. In addition, Szasz et al. (13), studying the phosphorylation of the calcium pump in isolated erythrocyte membranes, did not show a decrease in phosphorylation in the presence of EGTA; there also appears to be a shift in the molecular mass of the enzyme in the presence of EGTA. However, in neither of these papers are the membrane preparations solubilised with detergents, as is the case in the experiments reported here.

The data presented thus further corroborates previous work (1,4) indicating that higher plant plasma membranes contain a calmodulin-dependent ATPase which strongly resembles the erythrocyte calcium pumping ATPase (4). The intact enzyme forms a phosphorylated intermediate of M_r 140,000 both in microsome preparations and when purified, in a manner anticipated for an E_1-E_2 ion translocating ATPase. The enzyme clearly differs from the higher plant endoplasmic reticulum calcium-pumping ATPase, which forms a phosphorylated intermediate of M_r 96,000 (14). Higher plant cells therefore appear to resemble mammalian cells in having two E_1-E_2 calcium pumping ATPases located in the plasma membrane and endoplasmic reticulum.

REFERENCES

1) Dieter, P. and Marmé, D. (1981) FEBS Lett. 125, 245-248.
2) Robinson, C., Larsson, C. and Buckhout, T.J. (1988) Physiologia Plantarum 72, 177-184.
3) Rasi-Caldogno, F., Pugliarello, M.C. and DeMichelis, M.I. (1987) Plant Physiology 83, 994-1000.
4) Briars, S.-A., Kessler, F. and Evans, D.E. (1988) Planta, in press.
5) Evans, D.E. (1988) Cell Biology International Reports 12, 383-396.
6) Rega, A.F. and Garrahan, P.J. (1975) J. Membrane Biol. 22, 313-317.
7) Wuytack, F., Raeymaekers, L., De Schutter, G. and Casteels, R. (1982) Biochim. Biophys. Acta 693, 45-52.
8) Niggli, V., Penniston, J.T. and Carafoli, E. (1979) J. Biol. Chem. 254, 9955-9958.
9) Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.
10) Gorg, A., Postel, J., Weser, H.W. and Boeken, W.H. (1985) Science Tools 32, 5-9.
11) Carafoli, E. and Zurini, M. (1982) Biochim. Biophys. Acta 683, 279-301.
12) Knauf, P.A., Proverbio, F. and Hoffman, J.F. (1974) J. Gen. Physiol. 63, 324-336.
13) Szasz, I., Hasitz, M., Sarkadi, B. and Gardos, G. (1978) Mol. Cell. Biochem. 22, 147-152.
14) Giannini, J.L., Gildensoph, L.H., Reynolds-Niesman, I. and Briskin, D.P. (1987) Plant Physiology 85, 1129-1136.

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Short communication

The calmodulin-stimulated ATPase of maize coleoptiles is a 140000-M_r polypeptide

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Abstract. The calmodulin-stimulated ATPase of maize (*Zea mays* L.) coleoptiles has been purified by calcium-dependent binding to a calmodulin affinity column. In the presence of protease inhibitors (phenylmethylsulfonylfluoride and chymostatin) a polypeptide of relative molecular mass (M_r) 140000 (±10000) is obtained on sodium-dodecylsulphate polyacrylamide gels. This polypeptide is recognised specifically by an affinity-purified polyclonal antibody to mammalian calmodulin-stimulated calcium-pumping ATPases and is of similar M_r to the erythrocyte-membrane calcium pump (138000 M_r).

Key words. Calmodulin-stimulated ATPase – Plasma membrane (Ca^{2+} transport) – *Zea* (Ca^{2+} transport)

Calcium transport and related ATPase activity in higher-plant cells has recently been reviewed (Evans 1988). The presence of a calmodulin-stimulated ($Ca^{2+}-Mg^{2+}$)-ATPase in plasma membrane or microsomes extracted from dark-grown shoots and light-grown leaves of *Zea mays* L. has been previously demonstrated (Dieter and Marmé 1981; Robinson et al. 1988). The enzyme may be purified by affinity chromatography on a calmodulin-Sepharose affinity column (Dieter and Marmé 1981), being specifically eluted by addition of ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA). The enzyme from dark-grown tissue shows properties expected of a plasma-membrane calmodulin-stimulated calcium-pumping ATPase and is maximally stimulated by 550 nM calmodulin to 107% of its basal rate in a crude membrane extract and 230% of its basal rate in purified extracts (Dieter and Marmé 1981). In this paper we report the results of experiments to determine the molecular size of the purified enzyme and demonstrate common antigenicity with the mammalian plasma-membrane calcium-pumping ATPase.

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Abbreviations. EGTA = ethylene glycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid; M_r = apparent molecular mass; SDS = sodium dodecyl sulphate

Plant material. Zea mays L. (cv. Golden Bantam) seeds were surface-sterilised in 1% (v/v) sodium hypochlorite for 20 min, rinsed in five changes of distilled water and soaked for 24 h in distilled water with aeration at 30° C. The seeds were transferred under green safelight to tubs containing perlite soaked in 0.1 mM $CaCl_2$ and 0.01% (w/v) "Dithane 945" (Pan Britannica Industries, Waltham Cross, Herts., UK). The tubs were sealed and kept at 30° C for a further 3.5 d in total darkness.

Enzyme purification. The enzyme was purified from 4.5-d-old dark-grown maize shoots by the method of Dieter and Marmé (1981), modified as follows: all buffers were freshly made in distilled, deionised water, and solutions containing Triton X-100 (Sigma, Poole, Dorset, UK) were sonicated before use. Phenylmethylsulfonyl fluoride (PMSF; 1 mM) was added to all buffers just before use. The microsome pellet from the first 48000·*g* centrifugation was resuspended in the same volume of buffer B1 [10% (w/v) sucrose, 25 mM 3-(N-morpholino)propanesulfonic acid (Mops) titrated with 25 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) to pH 7.5, 1 mM dithiothreitol (DTT) and 1 mM PMSF] and recentrifuged at 48000·*g* to produce a pellet of "washed" microsomes. Solubilised proteins were applied to a Sigma calmodulin-agarose affinity column, which was washed with buffer C [buffer B1, without DTT, but with 0.5 M NaCl, 5 mM $CaCl_2$ and 0.05% (w/v) Triton X-100] for 2 h at which time the absorbance of the eluent at 280 nm was zero. The eluent was brought to 20 mM $CaCl_2$ and assayed immediately for calmodulin-stimulated ATPase activity, or prepared for gel electrophoresis.

Protein assay. The protein content of the samples was determined by the method of Bensadoun and Weinstein (1976) based on that of Lowry et al. (1951) with bovine serum albumin (BSA) as standard.

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Gel electrophoresis and Western transfer. Samples of the calmodulin affinity-purified enzyme (50 μg protein) were concentrated by mixing with an equal volume of ice-cold buffer 1 [10% (w/v) trichloracetic acid (TCA), 0.1 mM ATP, 0.8 mM orthophosphoric acid]. The mixture was centrifuged at 13000·*g* for 5 min, and the pellet washed once in buffer 2 (5% TCA, 0.1 mM ATP, 1 mM phosphoric acid). The pellet was washed twice in distilled water, then taken up into a minimal volume of sodium dodecyl sulphate (SDS) sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10 mM DTT, 10% sucrose, 0.002% bromophenol blue, 5 mM PMSF and 500 μg/ml chymostatin; the protease inhibitors being added just before use). Washed microsome samples (100 μg protein) were prepared similarly but without TCA concentration and were incubated in SDS sample buffer for 3 h on ice prior to electrophoresis. Samples were centrifuged at 13 000·*g* for 2 min before loading onto 7.5% discontinuous SDS polyacrylamide gels (Laemmli 1970).

After electrophoresis at 30 V overnight, proteins were stained with Coomassie blue dye (0.025% Coomassie Brilliant Blue R in 50% methanol, 5% acetic acid). For immunoblotting, proteins from the SDS gels were electrophoretically transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, FRG; 0.2 μm pore size, grade BA 83). The transfer buffer was 25 mM Tris-HCl, pH 8.3, 192 mM glycine, 20% methanol, and transfer was carried out at 50 V for 3 h. Membranes were blocked for 3 h with 10% milk powder ('Easypints'; J. Sainsbury, London, UK) in phosphate-buffered saline (0.2 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4 and 0.5 mM KH_2PO_4) containing 0.02% sodium azide (PBSA) at room temperature. Subsequent additions were made in 0.5% milk powder in PBSA (milk-PBSA); between each stage the membranes were washed 3 × 5 min in milk-PBSA. The nitrocellulose membranes were incubated with gentle agitation for 16 h in primary antibody (97.1 raised against purified erythrocyte membrane Ca^{2+}-pumping ATPase, affinity purified and screened as described by Verma et al. 1984) diluted 10-fold in milk-PBSA or in milk-PBSA alone. This was followed by incubation with Janssen (Grove, Wantage, UK) blotting-grade AuroProbe polyvalent goat anti-rabbit gold-conjugated secondary antibodies for 24 h. The gold was enhanced using a Janssen silver enhancement kit according to the manufacturers' instructions.

Enzyme purification. Calmodulin-stimulated ATPase was retained by the affinity column and eluted specifically by EGTA. The results obtained (data not shown) were comparable to, or better than, those of Dieter and Marmé (1981). The resultant calmodulin-stimulated enzyme preparation had a specific activity of 342 nmol phosphate released·min^{-1}·(mg protein)$^{-1}$.

Gel electrophoresis and immunostaining. The result of electrophoresis of the purified polypeptide on 7.5% SDS-polyacrylamide gels is shown in Fig. 1 (lane b). A polypeptide of M_r 140000 (±10000) is evident, together with polypeptides of M_r 91 000, 77000-69000, 51000 and 40000. Polypeptides of M_r 76000-62000 are present alone when the enzyme is purified in the absence of protease inhibitors, and that at M_r 40000 became predominant after concentration of the sample using an Amicon (Stonehouse, Gloucester, UK) microconcentrator

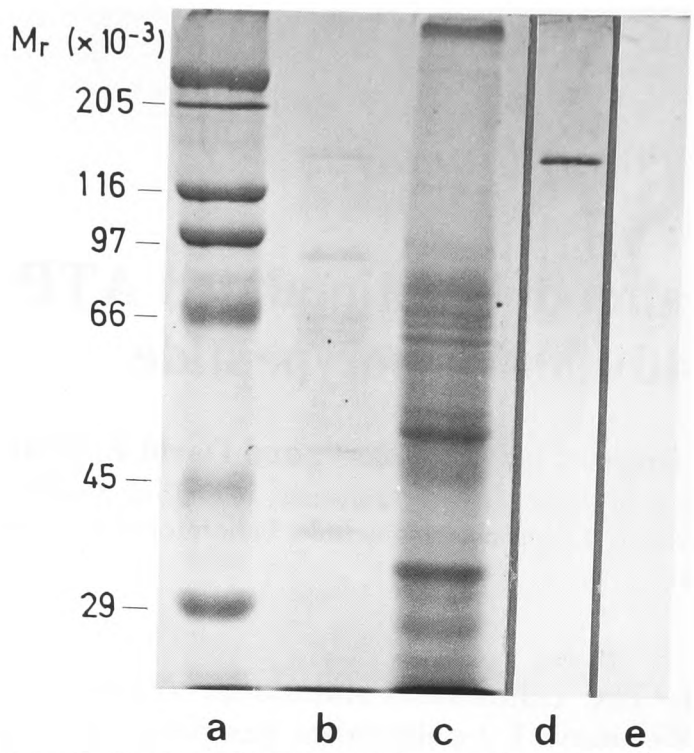


Fig. 1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblotting of microsomes and purified calmodulin-stimulated ATPase from dark-grown maize shoots. *Lanes a–c*, Coomassic-Blue-stained 7.5% SDS-polyacrylamide gel of M_r standards (*lane a*), microsomes (*lane c*) and purified calmodulin-stimulated ATPase (*lane b*). *Lanes d, e*, immunostaining of electrophoretically transferred purified enzyme. Polypeptides were transferred from a 7.5% SDS-polyacrylamide gel (as *lane b*) and immunostained using affinity-purified antibody 97.1. *lane d*, +antibody; *lane e*, control (-primary antibody)

(data not shown). It seems likely that they represent proteolysis products of the M_r 140000 polypeptide. Although partial contamination of the sample with other calmodulin-binding proteins cannot be eliminated at this stage, it is interesting to note that the calcium-pumping ATPase from mammalian erythrocyte membranes is also sensitive to proteolysis, forming polypeptides of M_r 90000, 81000-76000, 50000 and 42000 (Carafoli and Zurini 1982). Western transfer of material from the 7.5% SDS-polyacrylamide gel to nitrocellulose paper followed by immunostaining with the affinity-purified polyclonal antibody 97.1 raised to erythrocyte calcium-pumping ATPase resulted in the staining of a single band of M_r 140000 (Fig. 1, lane d), strongly indicating that this polypeptide is the higher-plant equivalent of the mammalian plasma-membrane calcium pump.

Previous work comparing the higher-plant calmodulin-stimulated ATPase with that from the erythrocyte membrane has indicated strong similarities with respect to calmodulin activation (Dieter and Marmé 1981; Robinson et al. 1988). In addition, calmodulin-stimulated calcium transport has been demonstrated in membrane preparations known to show calmodulin-stimulated ATP

hydrolysis (Dieter and Marmé 1980). The higher-plant enzyme has also been shown to be located in the plasma membrane (Robinson et al. 1988). The work described here indicates further similarities between the two enzymes. Common antigenicity is indicated by the fact that an antibody raised against the mammalian enzyme recognises the higher-plant polypeptide. The remarkable similarity in M_r between the two enzymes (mammalian erythrocyte, M_r 138000, Niggli et al. 1979) and the higher plant enzyme (M_r 140000) is also evident. The presence of polypeptides in gels of the purified enzyme from higher plants of similar M_r to proteolytic fragments from the mammalian enzyme may indicate further similarity.

It is interesting to note that after partial proteolysis, the erythrocyte enzyme yields a polypeptide of M_r approx. 76000 which is still active with respect to ATP hydrolysis, calcium transport and calmodulin stimulation (see Carafoli 1987). It has been suggested (Carafoli 1987) that the remaining 60000-M_r polypeptide contains regulatory sites. If this is so, the results of this study indicate that the plant enyzme has equivalent regulatory sites and the possibility that identical regulatory mechanisms in addition to calmodulin occur must be considered. Such regulation may involve phosphorylation by protein-kinase activity (demonstrated for a plant plasma-membrane proton-translocating ATPase by Schaller and Sussman (1988)).

Recently, Giannini et al. (1987) estimated the M_r of the calcium transporter from the endoplasmic reticulum of red beet to be 96000 on the basis of the formation of phosphorylated intermediates. The plasma-membrane calmodulin-stimulated ATPase thus appears to be a substantially larger enzyme, once again indicating the presence of regulatory sequences. This parallels the situation in mammalian tissue, where the calcium pump of the endoplasmic reticulum is of about 100000 M_r (MacLennan 1970).

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References

Bensadoun, A., Weinstein, D. (1976) Assay of proteins in the presence of interfering materials. Anal. Biochem. **70**, 241–250

Carafoli, E. (1987) Intracellular calcium homeostasis. Annu. Rev. Biochem. **56**, 395–433

Carafoli, E., Zurini, M. (1982) The Ca^{2+}-pumping ATPase of plasma membranes. Purification, reconstruction and properties. Biochim. Biophys. Acta **683**, 279–301

Dieter, P., Marmé, D. (1980) Calmodulin activation of plant microsomal Ca^{2+} uptake. Proc. Natl. Acad. Sci. USA **77**, 7311–7314

Dieter, P., Marmé, D. (1981) A calmodulin-dependent, microsomal ATPase from corn (*Zea mays* L.). FEBS Lett. **125**, 245–248

Evans, D.E. (1988) Regulation of cytoplasmic free calcium by plant cell membranes. Cell Biol. Int. Rep. **12**, 383–396

Giannini, J.L., Gildensoph, L.H., Reynolds-Niesman, I., Briskin, D.P. (1987) Calcium transport in sealed vesicles from red beet (*Beta vulgaris* L.) storage tissue. I. Characterization of a Ca^{2+}-pumping ATPase associated with the endoplasmic reticulum. Plant Physiol. **85**, 1129–1136

Laemmli, U.K. (1970) Cleavage of structural proteins using the assembly of the head of bacteriophage T4. Nature **227**, 680–685

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randal, R.J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. **193**, 265–275

MacLennan, D.H. (1970) Purification and properties of an adenosine triphosphatase from sarcoplasmic reticulum. J. Biol. Chem. **245**, 4508–4518

Niggli, V., Penniston, J.T., Carafoli, E. (1979) Purification of the $(Ca^{2+}-Mg^{2+})$-ATPase from human erythrocyte membranes using a calmodulin affinity column. J. Biol. Chem. **254**, 9955–9958

Robinson, C., Larsson, C., Buckhout, T.J. (1988) Identification of a calmodulin-stimulated $(Ca^{2+}-Mg^{2+})$-ATPase in a plasma membrane fraction isolated from maize (*Zea mays* L.) leaves. Physiol. Plant. **72**, 177–184

Schaller, G.E., Sussman, M.R. (1988) Phosphorylation of the plasma membrane H^+-ATPase of roots by a calcium-stimulated protein kinase. Planta **173**, 509–518

Verma, A.K., Penniston, J.T., Muallen, S., Lew, V. (1984) Effects of affinity-purified antibodies on the Ca^{2+} pumping ATPase of erythrocyte membranes. J. Bioenerg. Biomembr. **16**, 365–378

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