Characterization of a 5'-nucleotidase and a high affinity Ca2+-stimulated ATPase from microsomes from Zea mays coleoptiles

Stephen Gary Carter
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd
Part of the Biochemistry Commons

Recommended Citation
Carter, Stephen Gary, "Characterization of a 5'-nucleotidase and a high affinity Ca2+-stimulated ATPase from microsomes from Zea mays coleoptiles " (1983). Retrospective Theses and Dissertations. 8458.
https://lib.dr.iastate.edu/rtd/8458

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
INFORMATION TO USERS

This reproduction was made from a copy of a document sent to us for microfilming. While the most advanced technology has been used to photograph and reproduce this document, the quality of the reproduction is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help clarify markings or notations which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is “Missing Page(s)”. If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure complete continuity.

2. When an image on the film is obliterated with a round black mark, it is an indication of either blurred copy because of movement during exposure, duplicate copy, or copyrighted materials that should not have been filmed. For blurred pages, a good image of the page can be found in the adjacent frame. If copyrighted materials were deleted, a target note will appear listing the pages in the adjacent frame.

3. When a map, drawing or chart, etc., is part of the material being photographed, a definite method of "sectioning" the material has been followed. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again—beginning below the first row and continuing on until complete.

4. For illustrations that cannot be satisfactorily reproduced by xerographic means, photographic prints can be purchased at additional cost and inserted into your xerographic copy. These prints are available upon request from the Dissertations Customer Services Department.

5. Some pages in any document may have indistinct print. In all cases the best available copy has been filmed.
Carter, Stephen Gary

CHARACTERIZATION OF A 5'-NUCLEOTIDASE AND A HIGH AFFINITY CALCIUM ION - STIMULATED ATPASE FROM MICROSOMES FROM ZEA MAYS COLEOPTILES

Iowa State University

University Microfilms International 300 N. Zeeb Road, Ann Arbor, MI 48106

Ph.D. 1983
PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark \( \checkmark \).

1. Glossy photographs or pages \( \checkmark \)
2. Colored illustrations, paper or print 
3. Photographs with dark background 
4. Illustrations are poor copy 
5. Pages with black marks, not original copy 
6. Print shows through as there is text on both sides of page 
7. Indistinct, broken or small print on several pages 
8. Print exceeds margin requirements 
9. Tightly bound copy with print lost in spine 
10. Computer printout pages with indistinct print 
11. Page(s) \( \underline{\text{lacking when material received, and not available from school or author.}} \)
12. Page(s) \( \underline{\text{seem to be missing in numbering only as text follows.}} \)
13. Two pages numbered \( \underline{\text{Text follows.}} \)
14. Curling and wrinkled pages 
15. Other 

University Microfilms International
Characterization of a 5'-nucleotidase and a high affinity Ca\(^{2+}\)-stimulated ATPase from microsomes from *Zea mays* coleoptiles

by

Stephen Gary Carter

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Department: Biochemistry and Biophysics
Major: Molecular, Cellular, and Developmental Biology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1983
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABBREVIATIONS</td>
</tr>
<tr>
<td>GENERAL INTRODUCTION TO THE DISSERTATION</td>
</tr>
<tr>
<td>SECTION I. PURIFICATION AND CHARACTERIZATION OF A 5'-NUCLEOTIDASE FROM Zea mays SHOOT MICROSOMES</td>
</tr>
<tr>
<td>SUMMARY</td>
</tr>
<tr>
<td>INTRODUCTION</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
</tr>
<tr>
<td>RESULTS</td>
</tr>
<tr>
<td>DISCUSSION</td>
</tr>
<tr>
<td>REFERENCES</td>
</tr>
<tr>
<td>SECTION II. A HIGH AFFINITY (Ca^{2+} + Mg^{2+})-ATPase IN CORN COLEOPTILE PLASMA MEMBRANES</td>
</tr>
<tr>
<td>SUMMARY</td>
</tr>
<tr>
<td>INTRODUCTION</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
</tr>
<tr>
<td>RESULTS</td>
</tr>
<tr>
<td>DISCUSSION</td>
</tr>
<tr>
<td>REFERENCES</td>
</tr>
<tr>
<td>SECTION III. PARTIAL PURIFICATION AND CHARACTERIZATION OF A Ca^{2+}-STIMULATED ATPase FROM PLASMA MEMBRANES FROM Zea mays COLEOPTILES</td>
</tr>
<tr>
<td>SUMMARY</td>
</tr>
<tr>
<td>INTRODUCTION</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
</tr>
<tr>
<td>RESULTS</td>
</tr>
<tr>
<td>DISCUSSION</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Section I

Table 1. Purification flow chart of the 5'-nucleotidase 28
Table 2. 5'-Nucleotidase substrate specificity 30
Table 3. Effect of inhibitors on 5'-nucleotidase activity 31
Table 4. Effect of divalent cations and EGTA on 5'-nucleotidase activity 32

Section II

Table 1. Substrate specificity of the (Ca$^{2+}$ + Mg$^{2+}$)-ATPase 73
Table 2. Effect of various inhibitors on the corn plasma membrane (Ca$^{2+}$ + Mg$^{2+}$)-ATPase activity 74
Table 3. Effect of fluphenazine on the corn plasma membrane (Ca$^{2+}$ + Mg$^{2+}$)-ATPase activity 75

Section III

Table 1. Enrichment of Ca$^{2+}$-ATPase activity through the purification scheme 111
Table 2. Substrate specificity of the CM-Cellulose treated Ca$^{2+}$-ATPase 112
Table 3. Effect of MgCl$_2$ and calmodulin on the CM-Cellulose treated Ca$^{2+}$-ATPase activity 112
LIST OF FIGURES

Section I

Figure 1. Sephacryl S-200 column profiles of 5'-nucleotidase and K^+-ATPase activities after hexyl agarose column chromatography 33
Figure 2. A Laemmli SDS/PAGE of purified 5'-nucleotidase (3% stacking and 8% separating gels) and molecular weight standard curve 35
Figure 3. Ouchterlony double diffusion plate 37
Figure 4. Effect of sucrose dilaurate on the stability of the purified 5'-nucleotidase 39
Figure 5. The pH profile of the purified 5'-nucleotidase 41
Figure 6. Lineweaver-Burk plot of 5'-nucleotidase inhibition by increasing concentrations of cAMP (0, 10, 20, 40, 80 μM) using 5'-AMP as the substrate 43
Figure 7. Lineweaver-Burk plot of 5'-nucleotidase inhibition by increasing concentrations of adenosine (0, 50, 100, 150, 200 μM) using 5'-AMP as the substrate 45
Figure 8. Replot of the intercepts from the Lineweaver-Burk plot of the adenosine inhibition of the 5'-nucleotidase in Figure 7 47
Figure 9. Sucrose density gradient profile of the 80,000 x g microsome pellet 49
Figure 10. The effect of 5'-nucleotidase antiserum on the activity of the 5'-nucleotidase 51

Section II

Figure 1. Sucrose density gradient profile 76
Figure 2. Effect of added divalent cations on the plasma membrane ATPase activity 78
Figure 3. Effect of increasing ATP concentrations at varying Ca^{2+} concentrations on the plasma membrane ATPase activity

Figure 4. Time course of Pi production

Figure 5. pH profile of the Ca^{2+}-stimulated ATPase activity

Figure 6. Effect of sodium vanadate on the plasma membrane ATPase activity

Figure 7. Effect of increasing free Ca^{2+} concentrations on the plasma membrane (Ca^{2+} + Mg^{2+})-ATPase

Figure 8. Hill plot of data from Figure 7

Section III

Figure 1. Hexyl agarose column chromatography of Zwittergent-extracted corn shoot plasma membranes

Figure 2. CM-Cellulose column chromatography of hexyl agarose treated Ca^{2+}-ATPase, diluted with high salt buffer

Figure 3. Sephacryl S-200 column chromatography of Ca^{2+}-ATPase activity after being chromatographed on the CM-Cellulose column

Figure 4. The gel electrophoresis of Sephacryl S-200 treated Ca^{2+}-ATPase

Figure 5. Sephadex G-100 column chromatography of hexyl agarose treated Ca^{2+}-ATPase activity

Figure 6. pH profile of hexyl agarose high salt Ca^{2+}-ATPase activity

Figure 7. Effect of increasing concentrations of different divalent cations on the total ATPase activity from the CM-Cellulose column

Figure 8. Effect of increasing concentrations of monovalent cations on the Ca^{2+}-ATPase from the CM-Cellulose column

Figure 9. Lineweaver-Burk plot of the effect of increasing levels of Ca^{2+} on the hexyl agarose 'high salt' Ca^{2+}-ATPase
Figure 10. Lineweaver-Burk plot of the CM-Cellulose treated Ca\textsuperscript{2+}-stimulated ATPase activity in the presence (•) and absence (○) of 125 mM KCl

Figure 11. Effect of increasing concentrations of fluphenazine on Ca\textsuperscript{2+}-ATPase activity

Figure 12. The effect of increasing levels of ophiobolin A on Ca\textsuperscript{2+}-ATPase activity
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATPase</td>
<td>Adenosine triphosphatase</td>
</tr>
<tr>
<td>Bis-Tris</td>
<td>1,3-bis[tris(hydroxymethyl)methylamine] propane</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CDTA</td>
<td>trans-cyclohexane-1,2-diamine-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>DCCD</td>
<td>N,N'-dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DES</td>
<td>Diethylstilbestrol</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>2-DG</td>
<td>2-Deoxyglucose</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethyleneglycol-bis(β-amino-ethylether)N,N'-tetraacetic acid</td>
</tr>
<tr>
<td>Hepes</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid</td>
</tr>
<tr>
<td>MES</td>
<td>2-((N-morpholino) ethanesulfonic acid</td>
</tr>
<tr>
<td>NDP</td>
<td>Nucleoside diphosphate</td>
</tr>
<tr>
<td>NMP</td>
<td>Nucleoside monophosphate</td>
</tr>
<tr>
<td>NTP</td>
<td>Nucleoside triphosphate</td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>pNPP</td>
<td>p-Nitrophenylphosphate</td>
</tr>
<tr>
<td>SDS/PAGE</td>
<td>Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris-(hydroxymethyl)-aminoethane</td>
</tr>
<tr>
<td>Zwittergent 3,12</td>
<td>N-dodecyl-N,N'-dimethyl-3-ammonio-1-propane sulfonate</td>
</tr>
</tbody>
</table>
GENERAL INTRODUCTION TO THE DISSERTATION

This dissertation has been written according to the alternate format for dissertations and theses. There are three sections contained within this dissertation, each of which is a complete manuscript which has been submitted for publication in a refereed journal. Each section contains a summary of what is described in that section, followed by an introduction, materials and methods, results, discussion, and the references cited in the text. There is also a general introduction to the dissertation at the beginning, describing what is contained in the dissertation and a general summary at the end, describing the overall findings from the three sections of dissertation.

The first part of the dissertation is concerned with the purification and characterization of a 5'-nucleotidase activity from the microsomes of corn coleoptiles. An enzyme such as the 5'-nucleotidase may be very important to the physiology of the corn cell since it has been shown to interact with both cyclic and noncyclic nucleotides. A protein which can serve as an interface to these two nucleotide systems could have a great deal of control over how each is metabolized and as a result have a considerable influence on the cell physiology. The physical characterization of the enzyme (i.e., molecular weight estimation, quaternary structure determination, and carbohydrate content of the glycoprotein) have been described. Also, the enzymatic functions of the protein have been determined. The relationship between the enzymology of the protein and a possible in vivo function are discussed.

The second section of the dissertation involves the characteriza-
tion of a Ca\textsuperscript{2+}-stimulated, Mg\textsuperscript{2+}-dependent ATPase activity from the plasma membrane-enriched fraction of the microsomes of corn coleoptiles. The properties of the enzyme are consistent with an \textit{in vivo} function of maintaining the very low intracellular concentrations of Ca\textsuperscript{2+} found in the cytosol. As the result of some stimuli on the cell, Ca\textsuperscript{2+} levels become elevated which in turn activate a number of cellular processes, and when the Ca\textsuperscript{2+} levels return to the normally low concentrations, these processes are again inactivated. The cellular component, in animal cells, responsible for removing Ca\textsuperscript{2+} from the cytosol after a Ca\textsuperscript{2+}-mediated stimulation of the cell is a Ca\textsuperscript{2+}-ATPase. Although the system is well-characterized in animal cells, there is essentially no information present about higher plant Ca\textsuperscript{2+}-ATPase enzymes. The work described in this section of the dissertation is important to understanding how the higher plant Ca\textsuperscript{2+}-ATPase functions and as a starting point to the investigation of the regulation of Ca\textsuperscript{2+} levels in plant cells.

The third and final section of the dissertation deals with the solubilization of the Ca\textsuperscript{2+}-ATPase activity from the plasma membrane-enriched fraction of the corn coleoptile microsomes and the partial purification and characterization of that activity. This is important to the understanding of the properties of the Ca\textsuperscript{2+}-ATPase since in the partially purified state, the enzyme activity of interest is less hindered by other proteins which were previously associated with the Ca\textsuperscript{2+}-ATPase in the plasma membrane. As a result, the partially purified Ca\textsuperscript{2+}-ATPase may better reflect the properties of the enzyme responsible for the active transport of Ca\textsuperscript{2+} than the plasma membrane bound ATPase.
SECTION I.

PURIFICATION AND CHARACTERIZATION OF A 5'-NUCLEOTIDASE

FROM *Zea mays* SHOOT MICROSOMES

by

STEPHEN G. CARTER and CARL L. TIPTON
A 5'-nucleotidase from Zea mays seedling shoot microsomes has been purified 125-fold to apparent homogeneity. The enzyme is competitively inhibited by cAMP ($K_i = 5.2 \mu M$) and is also inhibited by adenosine in a noncompetitive manner ($K_i < 57 \mu M$). The inhibition by adenosine allowed us to distinguish the specific 5'-nucleotidase activity from that of nonspecific acid phosphatase activity in crude tissue homogenates. When an assay based on that observation is used, about half of the total 5'-nucleotidase activity in a crude homogenate is estimated to be associated with the microsomal membranes.

The microsomal enzyme has been solubilized and purified. Molecular weight estimates from gel filtration and from gel electrophoresis in sodium dodecylsulfate suggest that the enzyme is composed of two subunits of $M_r$ 24,500 and 25,500. The purified enzyme is specific for nucleoside monophosphates, with the activity assayed with p-nitrophenylphosphate, α- and β-glycerol phosphates and ribose-5-phosphate ranging from 0-3% of that with 5'-AMP. $K_m$ values for purine nucleotides are somewhat lower than for pyrimidine nucleotides: 5'-AMP, 57 $\mu M$; 5'-GMP, 57 $\mu M$; 5'-IMP, 81 $\mu M$; 5'-UMP, 200 $\mu M$; and 5'-CMP, 333 $\mu M$. Divalent cations were found to have no effect on the enzyme activity. Two cytokinins were tested as potential inhibitors but were not as effective as adenosine, and zeatin riboside-5'-phosphate did not serve as a good substrate indicating that the enzyme is not directly involved with phytohormone metabolism. A possible role for the enzyme in cyclic nucleotide metabolism and nucleotide pool size regulation is discussed.
INTRODUCTION

Membrane-bound 5'-nucleotidase (E.C.3.1.3.5) from animal tissue is a glycoprotein that has been shown to be involved in nucleoside transport across the plasma membrane (1-3) and in the regulation of the nucleotide pool size of the cell (4-7). The animal 5'-nucleotidase can exist in different forms and have different subcellular distribution, depending on the tissue studied. The best studied 5'-nucleotidase is the enzyme found in the plasma membranes of a variety of tissues (8-11). This has been shown to be an ecto-enzyme (i.e., an external catalytic site) (10, 12), the major function of which is to hydrolyze external nucleoside monophosphates to the corresponding nucleoside (13-15). The nucleoside is then transported into the cell via a nucleoside transport system (14, 16, 17) for rephosphorylation to the nucleotide. Another function of the ectoenzyme 5'-nucleotidase is in the production of adenosine, from extracellular nucleotides, to act as a local hormone to the surrounding tissue. Adenosine has been shown to be a potent regulator of vasodilation (14, 18, 19) and as a neuromodulator (20).

Stanley et al. (21) have demonstrated that 5'-nucleotidase activity in rat cells, normally associated with the plasma membrane, is also distributed in an intracellular membrane pool and that this activity is indistinguishable from that activity associated with the plasma membranes. This indicated that the 5'-nucleotidase activity from plasma membranes may be circulated through the cell through the process of internalization; however, the mechanism and the purpose of this process is not known.
A soluble, cytosolic form of 5'-nucleotidase has also been studied in animal cells (7, 13, 22). This enzyme appears to be involved with the regulation of the nucleotide pool size of the cell (7). As the high energy nucleotide pool sizes (NTP and NDP) drop as the result of an environmental stress (23) or in response to 2-deoxyglucose (2-DG) (15, 24), the nucleoside monophosphate pool size increases, unless there is a mechanism for the degradation of the nucleoside monophosphates to the nucleoside. Cells exposed to 2-DG have a sharp drop on the total nucleotide pool size (15, 24) and an increase in the concentration of nucleosides (i.e., adenosine) (15). The enzyme believed to be responsible for the hydrolysis of the nucleoside monophosphates to the nucleoside and inorganic phosphate is the cytosolic 5'-nucleotidase.

The bulk of the information obtained about 5'-nucleotidase activities has come from animal tissue sources; however, there have been a few reports dealing with 5'-nucleotidase activities from higher plant sources. Polya has described a soluble 5'-nucleotidase from potato tubers (25) and from wheat seedling leaves (26) both of which are regulated by cyclic nucleotides, and Chen and Kristopeit (27) have demonstrated the presence of two forms of 5'-nucleotidase activity from wheat germ which are believed to be involved with cytokinin metabolism.

In this report, we describe the purification and characterization of a 5'-nucleotidase activity from corn coleoptile microsomes and discuss a possible role for the enzyme in the regulation of the nucleotide pool size and the cyclic nucleotide metabolism of the cell.
MATERIALS AND METHODS

Two cultivars of *Zea mays* seed were used in the experiments presented in this paper. A hybrid, B73H* X Mo17H*, was obtained from the M. Brayton Seed Co., Ames, IA, and W64A seed was generously supplied by Dr. Charles Martinson, Department of Plant Pathology, Weed and Seed Sciences, Iowa State University.

All the compounds tested as substrates for the enzyme were obtained from Sigma Chemical Co., St. Louis, MO, with the exception of zeatin riboside-5'-phosphate which was the kind gift of Dr. Chong-maw Chen, Department of Life Sciences, University of Wisconsin-Parkside, Kenosha, WI. All the inhibitors tested were also from Sigma with the exception of adenosine which was obtained from Calbiochem-Behring Corp., San Diego, CA, as was the Zwittergent 3,12 detergent. Hexyl agarose was obtained from Miles-Yeda Laboratories, Elkhart, IN, and the Sephacryl S-200 was obtained from Pharmacia Fine Chemicals, Inc., Piscataway, NJ.

**Tissue Preparation**

Corn seeds were germinated at 28°C in the dark. After 3 days, the etiolated shoots were removed from the seedlings, rinsed four times with deionized water, weighed, and cut into 1-cm lengths. The shoot segments were then added to buffer A (0.25 M sucrose, 1.25 mM MgCl₂, 1.25 mM EDTA, pH 7.2 with Tris) at a ratio of 4 ml buffer/gram shoots. All subsequent steps were performed at 4°C.
Homogenization

The suspension was homogenized in a Polytron homogenizer (Brinkman Corp., Luzern, Switzerland) for 30s at setting '4' followed by 60s at setting '6'. Polyvinylpyrrolidone (0.25 g/gram fresh weight of shoots) was added and mixed thoroughly. The mixture was then filtered through 4 layers of cheesecloth to remove the bulk of the fibrous material before centrifugation.

Centrifugation

The filtrate was centrifuged at 1,465 x g for 10 minutes, the pellet discarded, and the supernate centrifuged at 12,000 x g for 20 minutes with the resulting pellet again being discarded. The 12,000 x g supernate was centrifuged at 80,000 x g for 90 minutes to pellet the microsomes. This pellet was resuspended in buffer A (0.1 ml/g shoots) and then repelleted at 80,000 x g for 90 minutes. The washed pellet was resuspended in 1 ml buffer A per 7 g shoots.

Detergent Extraction

Zwittergent 3,12 and KCl were added to give final concentrations of 1% (w/v) and 1 M, respectively. After 10 minutes at 20°C, the suspension was centrifuged at 80,000 x g for 90 minutes, and the supernate saved.
Hexyl Agarose Column Chromatography

The Zwittergent extract was dialyzed 6 hours against buffer A, then overnight against buffer B (0.25 M NaClO₄, 1.25 mM MgCl₂, 1.25 mM EDTA pH 8.0 with Tris). The dialysis retentate was applied to a hexyl agarose column (1.6 cm x 30 cm) equilibrated previously with buffer B. The column was then eluted with 50 ml buffer B. The 5′-nucleotidase was retained on the column during this elution but was eluted at a higher salt concentration (80 ml of buffer C: 2.0 M NaClO₄, 1.25 mM MgCl₂, 1.25 mM EDTA pH 8.0 with Tris).

Sephacryl S-200 Column Chromatography

The 5′-nucleotidase peak from the hexyl agarose column was concentrated to approximately 4 ml by using Aquacide II-A (Calbiochem-Behring Corp., San Diego, CA). The enzyme concentrate was dialyzed against buffer D (1 M NaCl, 1.25 mM MgCl₂, 1.25 mM EDTA pH 8.0 with Tris), then applied to a Sephacryl S-200 column (2.6 cm x 80 cm) equilibrated with buffer D.

The peak of 5′-nucleotidase activity eluted between the activity peaks of an acid phosphatase and a K⁺-ATPase, so that the pooled 5′-nucleotidase fractions were contaminated by the other two enzymes. The pooled 5-nucleotidase fractions were reconcentrated with Aquacide II-A and reapplied to the Sephacryl S-200 column. The 5′-nucleotidase pool obtained from the second Sephacryl column treatment was essentially free of K⁺-ATPase and acid phosphatase activity.
Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis (SDS/PAGE)

A portion of the 5'-nucleotidase after the second Sephacryl S-200 column was prepared for SDS/PAGE by precipitation with 5% (w/v) trichloroacetic acid and washed with cold ethanol. Slab gels of acrylamide (3% stacking, 10% separating) were prepared according to the method described by Laemmli (28).

Protein Assays

Protein was determined by using the trichloroacetic acid precipitation modification of the Lowry protein assay (29) or the Bradford protein assay (30).

Glycoprotein Carbohydrate Determination

The amount of carbohydrate associated with the 5'-nucleotidase was estimated by using the periodic acid/Schiff reaction described by Mantle and Allen (31).

Inorganic Phosphate Assays

The Fiske-SubbaRow phosphate assay (32) was used to measure the activity of the 5'-nucleotidase in the purification scheme up to the hexyl agarose step. After chromatography on the hexyl agarose column, the enzyme was assayed by a modification of the phosphate assay of Altmann et al. (33), developed in this laboratory (34). This method is about 10-fold more sensitive than the Fiske-SubbaRow method.
Enzyme Assay Conditions

5'-Nucleotidase

The assay for the 5'-nucleotidase contained 10 mM BisTris adjusted to pH 6.8 with HCl, 0.01% (w/v) sucrose dilaurate, 0.05-0.20 µg enzyme protein, and 200 µM 5'-AMP unless otherwise stated. All assays were incubated for periods of 1 hour or less at 37°C in a total volume of 1 ml.

K⁺-ATPase

The assay for the K⁺-ATPase contained 10 mM Hepes adjusted to pH 7.5 with imidazole, 20 µg BSA, +/- 250 mM KCl and 1 mM 5'-ATP. The enzyme was assayed at 37°C for 1 hour or less in a total volume of 1 ml. The activity reported is the increment due to the addition of KCl.

Acid phosphatase

The assay for the acid phosphatases contained 10 mM MES adjusted to pH 5.5 with Tris, and 200 µM p-nitrophenylphosphate. The enzyme was assayed at 37°C for 30 minutes or less in a total volume of 1 ml.

Sucrose Density Gradient Centrifugation

The 80,000 x g microsomal pellet was resuspended in 2 ml of 0.25 M sucrose, 1 mM Tris, pH 7.2 with MES. This suspension was floated on a linear (20-50%, w/w) sucrose gradient. This gradient was centrifuged in a SW27 rotor at 112,840 x g for 2 hours under a slow acceleration mode in a Beckman L-8 ultracentrifuge. The gradient was fractionated by puncturing the bottom of the tube and collecting drops.
Analysis of Kinetic Data

The kinetic parameters for the 5'-nucleotidase were determined by using the OMNITAB computer program of Siano, Zyskind, and Fromm (35).

Antibody Production

Polyclonal antibodies were produced against the purified 5'-nucleotidase in New Zealand white rabbits according to the following schedule. Four hundred µg of purified 5'-nucleotidase was injected as an emulsion with Freund's complete adjuvant, subcutaneously in the back of the rabbit. After one month, a booster injection of 200 µg of 5'-nucleotidase (in 0.9% NaCl, 0.1 M Hepes adjusted to pH 7.4 with imidazole) was injected in the ear vein. Nine days following the booster injection, the rabbit was bled and tested for the presence of 5'-nucleotidase antibodies.

Antibody Specificity

The specificity of the rabbit antiserum was determined using an Ouchterlony double diffusion test. The purified 5'-nucleotidase was tested against the immune and the preimmune rabbit serum.
RESULTS

The 5'-nucleotidase activity in extracts of corn seedlings was first noticed as an activity that accompanied a microsomal $K^+$-ATPase until the final step of the ATPase preparation (36). Consequently, it was possible to purify the nucleotidase to apparent homogeneity with minor modifications of the scheme used for the $K^+$-ATPase. Because of the large amounts of nonspecific phosphatase activity present in the seedling tissues, it was difficult to determine specific 5'-nucleotidase activity present before chromatography on the Sephacryl S-200 column, at which point the last of the phosphatase activity was resolved from the 5'-nucleotidase activity. Advantage was taken of the fact that 5'-nucleotidase is inhibited by adenosine (Tables 1 and 3), while nonspecific phosphatase activity is not affected (not shown), to estimate the amount of 5'-nucleotidase in the presence of nonspecific phosphatases. The 5'-nucleotidase activities shown in Table 1 are calculated from the differences in AMPase activity in the presence and absence of 2 mM adenosine.

In the 1,465 x g supernate, the hydrolysis of 5'-AMP is inhibited about 12% by 2 mM adenosine. This is a measure of the hydrolysis by the 5'-nucleotidase. Presumably the remaining 88% of the 5'-AMP hydrolysis is due to acid phosphatase activity. However, since the assay was performed at pH 6.8 (far from the optimal pH for an acid phosphatase), this is probably a low estimate of the amount of acid phosphatase present in this sample relative to 5'-nucleotidase. This method of estimating 5'-nucleotidase activity is subject to large relative errors when the
amount of phosphatase is much greater than the amount of 5'-nucleotidase, but it has allowed us to estimate the 5'-nucleotidase activity during the purification of the enzyme when other assay procedures were useless. Overall, 125-fold purification was achieved with 10% recovery of activity.

Molecular Characterization of the Purified 5'-Nucleotidase

From the position of elution of the 5'-nucleotidase from a calibrated Sephacryl S-200 column, the apparent molecular weight was calculated to be 49,000 +/- 6000 (Figure 1). However, when the purified enzyme was examined by SDS/PAGE, two bands appeared corresponding to molecular weights of 24,500 and 25,500 (Figure 2).

According to an estimate of the carbohydrate content of the enzyme by periodate-Schiff method of Mantle and Allen (31), the enzyme contains 40% (w/w) carbohydrate.

Purity of the 5'-Nucleotidase

The results of the SDS/PAGE of the 5'-nucleotidase preparation after a second treatment on the Sephacryl S-200 column shows the presence of two bands (Figure 2). Although this indicates that there are two different proteins present in the denatured 5'-nucleotidase preparation, the molecular weight estimation of the two bands, when added, is very close to the value of 49,000 +/- 6000 daltons for the native protein. This suggests that the purified 5'-nucleotidase may be composed of two subunits and has been purified to apparent homogeneity.

This preparation of enzyme was used for the production of polyclonal
antibodies. The results of an Ouchterlony diffusion plate using the anti-5'-nucleotidase antiserum and the purified 5'-nucleotidase protein is shown in Figure 3. One precipitin line is present between the 5'-nucleotidase well and the 5'-nucleotidase antiserum well, while there are no precipitin lines present between the 5'-nucleotidase and the preimmune serum wells. The presence of one precipitin line gives further indication of the homogeneity of the 5'-nucleotidase preparation.

Stability

Once the 5'-nucleotidase has been purified by the Sephacryl S-200 chromatography, it may be stored at -20°C without appreciable loss of enzymatic activity for a period of months. However, incubating the enzyme for variable lengths of time at 37°C before substrate addition results in a decline in the activity of the enzyme. The presence of sucrose dilaurate prevented the inactivation of the enzyme (Figure 4) and resulted in a linear time course of product production. Similar results were obtained with bovine serum albumin or Zwittergent 3,12 added to the enzyme. The rate of the reaction is proportional to enzyme concentration over a 10-fold range of protein concentrations (0.05-0.5 μg/ml).

pH Profile

A pH profile encompassing the maximum and minimum physiological values found in the plant cell as established by $^{31}$P NMR studies (37) is shown in Figure 5. There is no clear optimal pH for the enzyme,
although it is inhibited in the higher pH range. The remainder of the experiments reported here were done at pH 6.8.

Substrate Specificity

$K_m$ values for the enzyme indicate stronger affinity for the purine nucleoside monophosphates than for pyrimidine nucleotides (Table 2). The cytokinin derivative zeatin-5'-monophosphate is a relatively poor substrate, while the glycerol phosphates, ribose-5-phosphate and p-nitrophenylphosphate, are virtually inactive as substrates.

Effects of Various Inhibitors

A series of substrate and product analogues were tested with the 5'-nucleotidase for their ability to inhibit the activity of the enzyme (Table 3). The plant hormones, zeatin and kinetin, were tested and did not greatly affect the enzyme.

Adenosine and cAMP were among the most effective inhibitors tested. cAMP acted as a competitive inhibitor (Figure 6), with a $K_i$ of 5.2 $\mu$M as determined by analysis of replots of intercepts and slopes from the Lineweaver-Burk plot (38). Adenosine inhibited the 5'-nucleotidase in a noncompetitive manner (Figure 7). A replot of the $1/v$ intercepts vs. adenosine concentration (Figure 8) yields a concave upward curve, characteristic of a steady-state random Uni-Bi mechanism (38) from which one cannot extract an exact value for the $K_i$; however, the Lineweaver-Burk plot indicates that the inhibition constant for adenosine is less than the $K_m$ for the substrate used (5'-AMP, $K_m = 57$ $\mu$M).
Effect of Divalent Cations and EGTA

A number of divalent cations were tested for their influence on the 5'-nucleotidase. The results, as shown in Table 4, indicate that the enzyme is not affected significantly by the presence or absence of any of the metal ions tested. The addition of 1 mM EGTA gave an 18% inhibition of the enzymatic activity. This does not necessarily indicate a specific interaction of the chelating agent with a metal in the enzyme, but rather it may be an ionic interaction of the negatively charged EGTA molecule with a substrate binding site.

Association of the 5'-Nucleotidase with Membranes

5'-Nucleotidase activity bound to the microsomes accounts for approximately half of the total 5'-nucleotidase activity found in the corn shoot. This activity is not removed by washing the membranes with buffer A; however, upon treatment with detergent and KCl, the previously bound activity, along with the major portion of the total membrane protein, becomes solubilized. Subjecting washed microsomal membranes to continuous sucrose density gradient centrifugation (Figure 9) shows that the specific 5'-nucleotidase activity is distributed among all of the resolved membrane fractions. However, the major fraction of the activity is located in the region of the gradient thought to be associated with the tonoplast and the endoplasmic reticulum membranes (1.08-1.13 g/cc²) (39-42).
The Effect of 5'-Nucleotidase Antiserum on the Activity of the 5'-Nucleotidase

The effect of polyclonal antibodies, directed against the purified 5'-nucleotidase, on the activity of the 5'-nucleotidase is shown in Figure 10. These results show that a portion of the antibodies produced against the whole enzyme are able to inhibit the enzymatic activity. This antibody could be used in place of adenosine for the estimation of 5'-nucleotidase activity in the presence of phosphatase.
DISCUSSION

The 5'-nucleotidase activity from corn coleoptile microsomes has been purified 125-fold from the crude homogenate. The low number associated with the purification of the 5'-nucleotidase to apparent homogeneity could be the result of two possibilities: 1) the activity of the crude homogenate material has been overestimated or 2) the activity of the purified material has been underestimated. A likely explanation for an underestimation of the purified enzyme activity is that an activation factor, present in the crude homogenate, has been removed from the 5'-nucleotidase during the purification scheme. It is unclear what the actual situation is; however, the combination of a very low turn-over number for the 5'-nucleotidase (~ 5) and the unlikely prospect that the 5'-nucleotidase comprises 0.8% of the total protein in the cell indicates that the 'fold-purification' of the enzyme is suspect.

The 5'-nucleotidase from corn microsomes seems to resemble the 5'-nucleotidase activities from potato (43) and wheat seedling leaves (44). However, despite similarities, there are enough differences to state that they are different proteins. All three enzymes are inhibited by nucleosides and can be regulated by cyclic nucleotides through a competitive inhibition of the hydrolysis of the substrate, with similar affinities for the cyclic nucleotide. The corn and potato 5'-nucleotidase both have native molecular weights of approximately 50,000 daltons on a calibrated gel filtration column, and when subjected to SDS/PAGE, both proteins migrate to positions corresponding to molecular weights of approximately 25,000 daltons, indicating that two subunits compose the
native protein. However, after the above-mentioned properties, the 5'-nucleotidases from corn, potato, and wheat seedling leaves differ in a number of characteristics.

Although all three enzymes are noncompetitively inhibited by adenosine, the end product of 5'-AMP hydrolysis, the corn enzyme has at least an order of magnitude higher affinity for the nucleoside than do the other two enzymes. There is also a substantial difference in the substrate specificity between the enzymes. The corn microsomal 5'-nucleotidase is specific for nucleoside monophosphates, hydrolyzing p-nitrophenylphosphate and other nonspecific phosphatase substrates at a rate less than 3% that of the 5'-AMP hydrolysis. In comparison, the potato 5'-nucleotidase will hydrolyze p-nitrophenylphosphate at a faster rate (114%) than 5'-AMP. The possibility that this discrepancy between the two enzymes was the result of the different assay conditions used was tested. When the corn 5'-nucleotidase was assayed under the conditions used for the potato 5'-nucleotidase, the rate of p-nitrophenylphosphate hydrolysis was 23% that of 5'-AMP hydrolysis, which although different from our initial findings (3%), is still quite different from the potato enzyme. The potato and corn 5'-nucleotidases also seem to be purified from different subcellular fractions. The procedure for the isolation of the potato 5'-nucleotidase suggests that it is either a soluble protein or one which is loosely associated with a cytoplasmic membrane. The corn 5'-nucleotidase is clearly associated with the microsomal fraction of the cell, as demonstrated by sucrose density gradient centrifugation, and can be removed from the membranes only after treatment with detergent and KCl. Two final points in which the
corn enzyme is different from the potato enzyme are that the potato 5'-nucleotidase activity is significantly affected by divalent cations and cytokinins, but the corn nucleotidase is not. The conclusion concerning the 5'-nucleotidase reported here and the other two cyclic nucleotide-regulated 5'-nucleotidases from potato and wheat seedling leaves is that we have isolated an enzyme which in some aspects is very similar to the other two enzymes; however, it is clearly a unique enzyme with different properties and characteristics.

The molecular weight of the 5'-nucleotidase was estimated by gel filtration and SDS/PAGE. The relationship of the molecular weights corresponding to the two bands on the SDS/PAGE and the molecular weight estimate for the native enzyme indicates that the 5'-nucleotidase is composed of two similar, but different subunits. The addition of the molecular weight of each protein band on the SDS/PAGE gives a molecular weight estimate of 50,000 for a native enzyme composed of one subunit of each type. This value correlates to the estimate of $M_r = 49,000 \pm 6000$ for the native 5'-nucleotidase according to gel filtration. Although estimates of the molecular weight of glycoproteins by methods based on comparisons of Stokes radii are not as accurate as those of simple polypeptides (45), the plausible errors are not so large as to invalidate the conclusion that the enzyme is composed of two similarly sized subunits.

A possible role of the corn coleoptile 5'-nucleotidase in plant hormone metabolism was considered and subsequently rejected. Because a similar enzyme from wheat germ (27) seems to be involved in cytokinin metabolism, we tested zeatin riboside-5'-phosphate as a substrate and
found very low activity. In addition, the plant hormones dimethylallylpirine riboside and kinetin riboside were tested for their ability to inhibit the enzyme. The hormones were found to be much less effective inhibitors than is adenosine, the product of the 5'-AMP hydrolysis. Also, the low in vivo concentrations of the hormones would require very low $K_i$ values for such a role to be considered; therefore, we do not believe that this enzyme is directly involved in the phytohormone metabolism of the cell.

An attractive hypothesis for the in vivo function of the corn coleoptile 5'-nucleotidase is a role in the regulation of cellular metabolism by cAMP. The concentration of cAMP found in corn coleoptiles is approximately $10^{-7}$ M (46), which may be compared to the $K_i$ for the corn 5'-nucleotidase of $5.2 \times 10^{-6}$ M. Although the cAMP level reported for the corn cell is lower than the $K_i$ we have obtained, a higher concentration of cAMP may result from compartmentalization or transient increases in cAMP synthesis. This elevated level of cAMP could inhibit the 5'-nucleotidase to a significant extent, allowing a transient accumulation of nucleoside monophosphates. These nucleotides could be used as precursors for the synthesis of polymucleotides as suggested by Polya (44), in response to a phytohormonal stimulation for the induction of the synthesis of an enzyme (47). In this sense, the 5'-nucleotidase acts as a shut off valve for the nucleotide degradation pathway, enabling the cell to produce nucleic acids with energetically more economical substrate precursors (48-50).

A more obvious role for the enzyme is regulation of the level of nucleoside monophosphates in the cell. It may be a key enzyme in
determining whether a nucleoside monophosphate is degraded to a nucleoside and subsequently to the respective base, or rephosphorylated to the di- or tri-phosphate. As shown by Saglio and Pradet (51), the depletion of energy sources (e.g., soluble sugars) and the consequent drop in the respiration rate of plant cells result in a drop in the adenine nucleotide pool size in the cell, but the ratio of the high energy nucleotides (ATP and ADP) to the total nucleotide pool (ATP, ADP, AMP) remains constant (52). A mechanism for such an occurrence is to allow for the hydrolysis of the nucleoside monophosphates via 5'-nucleotidase action in coordination with the drop of the high energy nucleotides. This function is also supported by a report by Lin and Hanson (24) showing the action of 2-deoxyglucose (2-DG) and ethionine on corn root adenine nucleotide levels. The addition of 10 mM 2-DG resulted in a drop in ATP and ADP levels (47 and 24 mmoles/g fresh weight, respectively), while the AMP levels increased only 8 mmoles/g fresh weight. To test whether the 5'-nucleotidase from corn coleoptiles had a role in the nucleotide pool size reduction, we attempted to inhibit the 5'-nucleotidase in vivo. 8-Azidoadenosine, synthesized according to a modified procedure from Schmidt et al. (53), and 8-bromoadenosine were used as inhibitors in these studies. Inhibition of the purified enzyme by these compounds (Table 3) show essentially total inhibition of the enzyme activity at 2 mM inhibitor concentration. However, experiments attempting to inhibit the 5'-nucleotidase in vivo were inconclusive. After treating the corn tissue with 2-DG and the inhibitors, we did not see an effect by the inhibitors on the nucleotide pools. This could mean that the inhibitors did not penetrate the cell, or it could mean that the enzyme
does not have a key role in controlling the nucleotide pool size. Additional experimentation from another approach will be required to obtain an answer to the question of the role of the membrane-bound 5'-nucleotidase in the plant cell.
REFERENCES


Table 1. Purification flow chart of the 5'-nucleotidase

<table>
<thead>
<tr>
<th>Isolation step</th>
<th>Total activity - adenosine (μmoles Pi/h)</th>
<th>Total activity + 2 mM adenosine (μmoles Pi/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,465 x g supernate</td>
<td>1,412</td>
<td>1,234</td>
</tr>
<tr>
<td>12,000 x g supernate</td>
<td>1,283</td>
<td>1,047</td>
</tr>
<tr>
<td>Microsome suspension</td>
<td>418</td>
<td>308</td>
</tr>
<tr>
<td>Detergent extraction</td>
<td>251</td>
<td>102</td>
</tr>
<tr>
<td>Hexyl agarose</td>
<td>76</td>
<td>22</td>
</tr>
<tr>
<td>1st Sephacryl S-200 column</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td>2nd Sephacryl S-200 column</td>
<td>22</td>
<td>5</td>
</tr>
<tr>
<td>Total 5'-nucleotidase activity (μmoles Pi/h)</td>
<td>Specific activity (μmoles Pi/h/mg)</td>
<td>Fold purification</td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>----------------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>178</td>
<td>0.35</td>
<td>-</td>
</tr>
<tr>
<td>236</td>
<td>0.57</td>
<td>1.6</td>
</tr>
<tr>
<td>110</td>
<td>0.77</td>
<td>2</td>
</tr>
<tr>
<td>149</td>
<td>2.5</td>
<td>7</td>
</tr>
<tr>
<td>54</td>
<td>5.7</td>
<td>16</td>
</tr>
<tr>
<td>25</td>
<td>29</td>
<td>81</td>
</tr>
<tr>
<td>17</td>
<td>44</td>
<td>125</td>
</tr>
</tbody>
</table>
Table 2. 5'-Nucleotidase substrate specificity

<table>
<thead>
<tr>
<th>200 μM substrate</th>
<th>Relative activity</th>
<th>Kₘ values</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'AMP</td>
<td>100</td>
<td>57.7 μM</td>
</tr>
<tr>
<td>5'GMP</td>
<td>116</td>
<td>57.1 μM</td>
</tr>
<tr>
<td>5'CMP</td>
<td>46</td>
<td>333.3 μM</td>
</tr>
<tr>
<td>5'UMP</td>
<td>71</td>
<td>200.0 μM</td>
</tr>
<tr>
<td>5'IMP</td>
<td>99</td>
<td>81.6 μM</td>
</tr>
<tr>
<td>5'ATP</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3'AMP</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>cAMP</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>p-Nitrophenylphosphate</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Ribose-5-phosphate</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>β-Glycerol phosphate</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>α-Glycerol phosphate</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Zeatin riboside 5'-phosphate</td>
<td>17</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Effect of inhibitors on 5'-nucleotidase activity

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>200 μM adenosine</td>
<td>53</td>
</tr>
<tr>
<td>1 mM adenosine</td>
<td>22</td>
</tr>
<tr>
<td>2 mM adenosine</td>
<td>18</td>
</tr>
<tr>
<td>200 μM 6-chloropurine riboside</td>
<td>57</td>
</tr>
<tr>
<td>1 mM 6-chloropurine riboside</td>
<td>10</td>
</tr>
<tr>
<td>200 μM 6-mercaptopurine riboside</td>
<td>91</td>
</tr>
<tr>
<td>1 mM 6-mercaptopurine riboside</td>
<td>59</td>
</tr>
<tr>
<td>50 μM cAMP</td>
<td>34</td>
</tr>
<tr>
<td>100 μM cAMP</td>
<td>26</td>
</tr>
<tr>
<td>150 μM cAMP</td>
<td>27</td>
</tr>
<tr>
<td>200 μM cAMP</td>
<td>24</td>
</tr>
<tr>
<td>50 μM adenosine-5'-sulfate</td>
<td>88</td>
</tr>
<tr>
<td>100 μM adenosine-5'-sulfate</td>
<td>83</td>
</tr>
<tr>
<td>150 μM adenosine-5'-sulfate</td>
<td>83</td>
</tr>
<tr>
<td>200 μM adenosine-5'-sulfate</td>
<td>79</td>
</tr>
<tr>
<td>50 μM 6-γ-γ-dimethylallylaminopurine riboside</td>
<td>89</td>
</tr>
<tr>
<td>100 μM 6-γ-γ-dimethylallylaminopurine riboside</td>
<td>79</td>
</tr>
<tr>
<td>150 μM 6-γ-γ-dimethylallylaminopurine riboside</td>
<td>72</td>
</tr>
<tr>
<td>200 μM 6-γ-γ-dimethylallylaminopurine riboside</td>
<td>64</td>
</tr>
<tr>
<td>10% dimethylsulfoxide</td>
<td>77</td>
</tr>
<tr>
<td>50% dimethylsulfoxide</td>
<td>22</td>
</tr>
<tr>
<td>2 mM 8-bromoadenosine</td>
<td>4</td>
</tr>
<tr>
<td>2 mM 8-azidoadenosine</td>
<td>8</td>
</tr>
<tr>
<td>200 μM ATP</td>
<td>88</td>
</tr>
<tr>
<td>200 μM kinetin riboside</td>
<td>64</td>
</tr>
</tbody>
</table>
## Table 4. Effect of divalent cations and EGTA on 5'-nucleotidase activity

<table>
<thead>
<tr>
<th>Sample</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>100 µM MnCl₂</td>
<td>102</td>
</tr>
<tr>
<td>100 µM MgCl₂</td>
<td>101</td>
</tr>
<tr>
<td>100 µM CoCl₂</td>
<td>100</td>
</tr>
<tr>
<td>100 µM ZnSO₄</td>
<td>98</td>
</tr>
<tr>
<td>100 µM FeSO₄</td>
<td>98</td>
</tr>
<tr>
<td>100 µM CaCl₂</td>
<td>98</td>
</tr>
<tr>
<td>100 µM NiSO₄</td>
<td>97</td>
</tr>
<tr>
<td>2 mM MgCl₂</td>
<td>99</td>
</tr>
<tr>
<td>1.0 mM EGTA</td>
<td>82</td>
</tr>
</tbody>
</table>
Figure 1. *Sephacryl S-200 column profiles of 5'-nucleotidase and $K^+$-ATPase activities after hexyl agarose column chromatography.* A 6 ml sample of protein eluted from the hexyl agarose column was applied to the Sephacryl S-200 column (2.6 x 80 cm), eluted with buffer D (see Materials and Methods section) at a flow rate of 0.77 ml/min. 5'-nucleotidase ($\bullet$), $K^+$-ATPase ($\circ$), acid phosphatase ($\odot$), $A_{280nm}$ ($\Delta$).
Figure 2. A Laemmli SDS/PAGE of purified 5'-nucleotidase (3% stacking and 8% separating gels) and molecular weight standard curve. A Coomassie blue protein stain of the 5'-nucleotidase enzyme preparation following the second Sephacryl S-200 column step in the purification scheme. Due to the poor staining properties of the 5'-nucleotidase with Coomassie blue, 50 µg of protein was applied to the gel and electro-phoresed. The molecular weight standard curve on the above gel used the following proteins as standards: 1) Albumin, Bovine (68,000), 2) Albumin, Chicken (45,000), 3) Glyceraldehyde-3-Phosphate Dehydrogenase (36,000), 4) Carbonic Anhydrase (29,000), 5) Trypsinogen (24,000). The two arrows on the standard curve indicate the relative positioning of the two bands from the 5'-nucleotidase preparation.
Figure 3. Ouchterlonv double diffusion plate. The specificity of the 5'-nucleotidase antiserum is shown using a double diffusion gel (1% agarose). IS, antiserum to the 5'-nucleotidase; PS, preimmune serum; N, purified 5'-nucleotidase
Figure 4. **Effect of sucrose dilaurate on the stability of the purified 5'-nucleotidase.** Purified 5'-nucleotidase was pre-incubated at 37°C for various periods of time with (A) and without (B) 0.01% sucrose dilaurate present after which 200 μM AMP was added. Assay time was 30 min.
Figure 5. The pH profile of the purified 5'-nucleotidase. Buffers used: pH 5.6, 10 mM MES-imidazole; pH 6.0-6.8, 10 mM Bis-Tris-HCl; pH 6.8-8.0, 10 mM Hepes-imidazole.
$\mu$ moles Pi/hr/mg.
Figure 6. Lineweaver-Burk plot of 5'-nucleotidase inhibition by increasing concentrations of cAMP (0, 10, 20, 40, 80 µM) using 5'-AMP as the substrate
Figure 7. Lineweaver-Burk plot of 5'-nucleotidase inhibition by increasing concentrations of adenosine (0, 50, 100, 150, 200 μM) using 5'-AMP as the substrate
Figure 8. Replot of the intercepts from the Lineweaver-Burk plot of the adenosine inhibition of the 5'-nucleotidase in Figure 7.
Figure 9. Sucrose density gradient profile of the 80,000 x g microsome pellet. Top panel: (o), sucrose concentration; (●) A550nm turbidity. Bottom panel: (●) specific 5'-nucleotidase activity.
Figure 10. The effect of 5'-nucleotidase antiserum on the activity of the 5'-nucleotidase. Increasing amounts of whole serum were added to a 5'-nucleotidase assay mixture, incubated at 25°C for 20 minutes prior to adding 5'-AMP, then incubated at 37°C and assayed as usual.
SECTION II.
A HIGH AFFINITY (Ca$^{2+}$ + Mg$^{2+}$)-ATPase IN
CORN COLEOPTILE PLASMA MEMBRANES

by

STEPHEN G. CARTER and CARL L. TIPTON
SUMMARY

A Ca\(^{2+}\)-stimulated, Mg\(^{2+}\)-dependent ATPase ((Ca\(^{2+}\) + Mg\(^{2+}\))ATPase) activity has been observed in a plasma membrane-enriched fraction of corn coleoptile microsomes. The Ca\(^{2+}\)-stimulated ATPase activity has a pH optimum at 6.5. The order of substrate specificity is ATP > UTP > GTP > CTP >> pNPP. The preference of the ATPase for divalent cations used for stimulating the enzyme is as follows: Ca\(^{2+}\) > Mg\(^{2+}\) > Co\(^{2+}\) > Mn\(^{2+}\) > Ni\(^{2+}\) > Zn\(^{2+}\). The enzyme activity is not sensitive to oligomycin, sodium azide, sodium vanadate, or sodium molybdate but it is inhibited by DCCD and DES. An analysis of the activation vs. Ca\(^{2+}\) concentration profile indicates the affinity constant for Ca\(^{2+}\) to be 0.2 \(\mu\)M in the presence of 14 \(\mu\)M Mg\(^{2+}\). However, in the absence of Mg\(^{2+}\), the Ca\(^{2+}\)-stimulated activity is abolished, indicating the requirement for Mg\(^{2+}\) by the ATPase. Fluphenazine, an inhibitor of many calmodulin-stimulated processes, also inhibits the plasma membrane (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase from corn approximately 60% at 50 \(\mu\)M, but the addition of exogeneous calmodulin to the enzyme does not activate it further. The comparison with animal (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPases is discussed.
INTRODUCTION

The regulation of the intracellular concentration of Ca\(^{2+}\) is critical for the normal growth and functioning of all eukaryotes. Ca\(^{2+}\) has been implicated in such important processes as cell growth, division, mobility, and secretion (1, 2). The mechanism by which Ca\(^{2+}\) elicits physiologic responses is still not fully understood, although since the discovery of calmodulin (3) and calmodulin-like calcium binding proteins (4), rapid advances have been made in this area. Ca\(^{2+}\) seems to be involved in cellular metabolism as a second messenger, in a role similar to that of cAMP. In general, the normally low intracellular concentration of Ca\(^{2+}\) (10\(^{-7}\) - 10\(^{-8}\) M) (5, 6, 7) increases as the result of an external event (i.e., hormonal stimulation) (8). The increased Ca\(^{2+}\) binds to and activates a calcium binding protein which in turn activates one or more enzymes (9). For the cell to maintain a high degree of sensitivity to such fast acting, transient events, there must be an effective means of removing temporarily high levels of Ca\(^{2+}\) from the cytoplasm to bring the system back to an inactivated state. The process most likely to mediate this is the active transport of Ca\(^{2+}\) across a cellular membrane by a Ca\(^{2+}\)-activated ATPase. This may be across the membranes of organelles (i.e., endoplasmic reticulum, or in plants, the tonoplast) for the sequestration of the Ca\(^{2+}\), or across the plasma membrane for the extrusion of Ca\(^{2+}\) from the cell.

There has been a large number of papers in recent years dealing with the characterization of plasma membrane-bound, Ca\(^{2+}\)-activated ATPase and subsequently the solubilization and isolation of the enzymes for
study in greater detail. The best characterized enzyme is that from erythrocyte ghosts (10), but recently, there have been notable advances made with plasma membranes from other tissues (i.e., liver and adipose) (11, 12). Ca\textsuperscript{2+}-activated ATPases from various animal tissues are quite similar, but Ca\textsuperscript{2+}-ATPases from higher plants have not been well enough characterized to allow comparison. Although there have been reports of an ATP-dependent Ca\textsuperscript{2+} transport process in plant plasma membranes (13, 14) and a calmodulin-activated Ca\textsuperscript{2+} uptake by plasma membranes (15), there has been very little characterization of the enzymes responsible for these interesting observations. Dieter and Marme (16) have isolated a calmodulin-dependent microsomal ATPase from the plasma membranes of corn coleoptiles, but they did not report much characterization of the solubilized enzyme and even less information about the membrane-bound form of the enzyme.

In this paper, we describe the characterization of a Ca\textsuperscript{2+}-activated Mg\textsuperscript{2+}-dependent ATPase activity from the plasma membranes of corn coleoptiles. The characterization of the enzyme is extensive enough to allow a comparison to be made between the (Ca\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPase from a higher plant source and the already well-characterized erythrocyte and adipocyte plasma membrane (Ca\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPases.
MATERIALS AND METHODS

Corn seedlings (B73H × Mo17H+) were grown at 28°C in the dark for three days. The etiolated shoots were removed from the seedlings with a razor blade, washed with deionized water, and weighed. The shoot segments were added to 0.25 M sucrose, 20 mM imidazole, adjusted to pH 7.2 with HCl (homogenization buffer) (4 ml/g shoots). The shoots were homogenized in a Waring blender, the crudely homogenized material was then transferred to a Polytron homogenizer (Brinkman Corp., Luzern, Switzerland) for 30s at setting '4' followed by 60s at setting '6'. Polyvinylpyrrolidone (0.25 g/g shoots) was added and thoroughly mixed. The homogenate was filtered through one layer of Miracloth (Calbiochem-Behring Corp., San Diego, CA) to remove the bulk of the fibrous material. The filtrate was centrifuged at 16,000 x g for 30 minutes in a Sorvall GSA rotor. The supernate was centrifuged in a Beckman 45 Ti rotor at 80,000 x g for 30 minutes to obtain the microsomal pellet. The pellet was resuspended in the homogenization buffer and layered on a continuous sucrose density gradient (20-50%, w/w) in 20 mM imidazole-HCl, pH 7.2. The gradient was centrifuged at 112,840 x g for 2 hours, using the slow acceleration mode on a Beckman L-8 centrifuge, and was fractionated by puncturing the bottom of the tube and collecting drops.

The sucrose concentrations of the gradient fractions were determined by using a Bausch and Lomb refractometer. The bands of membranes were detected by measuring the turbidity of each fraction at 550 nm. Enzymes were assayed under the following conditions, unless otherwise stated: (Mg^{2+} + K^+) - ATPase, 33 mM MES pH 6.0 with Tris, 50 mM KCl,
1.5 mM MgCl₂, 3 mM ATP (17); (Ca²⁺ + Mg²⁺)-ATPase, 10 mM Hepes pH 6.5 with imidazole, with and without 100 μM CaCl₂, 100 μM ATP. There were no chelating reagents added to the assay mixtures unless stated.

The protein determinations were made using a TCA precipitation variation of the procedure of Lowry (18).

Inorganic phosphate formed in the ATPase assays was estimated as described earlier (19).

The levels of magnesium and calcium in the plasma membrane assay mixtures were estimated by atomic absorption spectrophotometry, with a Varian Techtron Model 1200 atomic absorption spectrophotometer, with a combination Ca²⁺/Mg²⁺/Ni²⁺ lamp source.

Calculations of the free divalent metal ions in the assay mixture were performed according to equations from Pershadsingh and McDonald (12). The final form of the equation used is as follows:

\[
[\text{Me}] = \text{total divalent ion concentration},
\]
\[
[L] = \text{total ligand concentration}, \text{ and}
\]
\[
K' = \text{association constant calculated for the Me-L complex at pH 6.5}.
\]

\[
[\text{MeL}] = \left(\frac{[\text{Me}]+[L]+(1/K')}{2}\right) - \sqrt{\left(\frac{[\text{Me}]+[L]+(1/K')}{2}\right)^2 - ([\text{Me}] \times [L])}
\]
The following apparent association constants have been calculated for pH 6.5, for the following ligand-metal ion combinations, using the true association constants from Pershadsingh and McDonald (12).

<table>
<thead>
<tr>
<th>Ion</th>
<th>EGTA</th>
<th>CDTA</th>
<th>ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca$^{2+}$</td>
<td>5.85</td>
<td>5.86</td>
<td>3.41</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>0.06</td>
<td>5.33</td>
<td>4.09</td>
</tr>
</tbody>
</table>
RESULTS

Previous attempts to study Ca\(^{2+}\)-stimulated ATPase activity in the plasma membranes from higher plants have been complicated by the presence of a Mg\(^{2+}\)-dependent, K\(^{+}\)-stimulated ATPase (Mg\(^{2+}\) + K\(^{+}\)-ATPase), in the plasma membranes in quantities which hindered the detection of the lower activity of the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase. To circumvent this problem, we have assayed for the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase activity in the plasma membranes under conditions which suppress the activity of the (Mg\(^{2+}\) + K\(^{+}\))-ATPase and at the same time give an optimal response from the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase. The conditions used to suppress the (Mg\(^{2+}\) + K\(^{+}\))-ATPase are low levels of ATP (100 \(\mu\)M) and no added Mg\(^{2+}\) or K\(^{+}\) at pH 6.5.

Distribution of the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase on a Continuous Sucrose Density Gradient

The 80,000 \(\times\) g microsome fraction was centrifuged on a sucrose density gradient to resolve the various membrane components. After fractionating and assaying the gradient, (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase activity was found to be associated with two peaks of membranes which migrated close together on the gradient, both of which have been shown to be enriched in plasma membranes (20, 21) (Figure 1). The two peaks of membranes with (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase activity were pooled (\(\rho\) 1.14-1.19 g/cc\(^2\)) and subsequently used as the plasma membrane-enriched fraction of the microsomes. The gradient fractions were also assayed for the presence of the (Mg\(^{2+}\) + K\(^{+}\))-ATPase, which has been demonstrated to be associated with the plasma membrane-enriched region of the microsomes.
(17, 22). The \((\text{Mg}^{2+} + \text{K}^+)\)-ATPase activity was associated with the same two membrane peaks as the \((\text{Ca}^{2+} + \text{Mg}^{2+})\)-ATPase on the gradient (not shown) giving further indication that the \((\text{Ca}^{2+} + \text{Mg}^{2+})\)-ATPase is a plasma membrane-bound ATPase.

### Substrate Specificity

In the absence of added \(\text{Ca}^{2+}\), the nucleoside triphosphates and pNPP are hydrolyzed at very similar rates (Table 1). Stimulation by added \(\text{Ca}^{2+}\) is nearly two-fold with ATP as the substrate and the rate decreases in the order UTP, GTP, CTP, pNPP.

### Divalent Cation Specificity

The membrane bound ATPase activity was tested for specificity, with respect to the ability of different divalent cations, at varying concentrations, to stimulate the enzyme. Endogenous levels of \(\text{Mg}^{2+}\) and \(\text{Ca}^{2+}\) in the assay mixture, determined by atomic absorption spectrophotometry, were 14.8 \(\mu\text{M}\) and 12.0 \(\mu\text{M}\), respectively. The results in Figure 2 show some stimulation by additional \(\text{Ca}^{2+}\) and \(\text{Mg}^{2+}\). \(\text{Co}^{2+}\) and \(\text{Mn}^{2+}\) are almost as effective at low concentrations while \(\text{Ni}^{2+}\) is nearly ineffective and \(\text{Zn}^{2+}\) is quite inhibitory at 200 \(\mu\text{M}\).

### Relationship of ATPase Activity to \(\text{Ca}^{2+}\) and ATP Concentrations

There is a large amount of ATPase activity associated with the membrane preparations in the absence of added \(\text{Ca}^{2+}\) (Figure 3). The
stimulation due to added Ca\(^{2+}\) saturates at 50-100 \(\mu\)M at all ATP concentrations tested. The magnitude of the Ca\(^{2+}\) stimulation does not increase with increasing ATP concentrations. It is likely that at least two ATPase activities are present, one Ca\(^{2+}\)-dependent and the other(s) Ca\(^{2+}\)-independent. To minimize interference from other ATPases in the plasma membranes, the Ca\(^{2+}\)-stimulated activity was assayed at 100 \(\mu\)M ATP, and 100 \(\mu\)M Ca\(^{2+}\). Under these conditions, the activity was linear with time at 37°C for at least one hour (Figure 4).

**pH Profile**

Figure 5 shows a peak of activity at pH 6.5 and a second smaller, but pronounced peak at pH 8.0. pH 6.5 was used for the standard assay of the enzyme.

**Effect of Inhibitor Substances on the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase Activity**

A variety of potential inhibitors were tested on the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase activity (Table 2, Figure 6). Oligomycin and sodium azide were ineffective. Sodium molybdate and sodium vanadate inhibited the Ca\(^{2+}\)-insensitive ATPase activity, but had little effect on the Ca\(^{2+}\)-stimulation. DCCD and DES were inhibitory to the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase at concentrations of \(10^{-4}\) to \(10^{-5}\) M.
Determination of the Ca$^{2+}$ Affinity Constant

To determine the affinity constant for Ca$^{2+}$ of the plasma membrane (Ca$^{2+}$ + Mg$^{2+}$)-ATPase, the chelating agent EGTA was used as a Ca buffer. The calculation of the free Ca$^{2+}$ concentration in the assay mixture was performed using variations of the equations and methods described by Pershadsingh and McDonald (12). The endogenous levels of Mg$^{2+}$ and Ca$^{2+}$ were incorporated into the calculations to determine the free divalent ion concentrations in the assay mixture in the presence of the chelating reagents (EGTA or CDTA) and ATP. The experiment performed to determine the $K_a$ of Ca$^{2+}$ for the plasma membrane (Ca$^{2+}$ + Mg$^{2+}$)-ATPase is shown in Figure 7. The $K_a$ value obtained from this curve is 0.2 $\mu$M Ca$^{2+}$, which is well within the reported range of Ca$^{2+}$ levels in plant cells (23).

Requirement for Magnesium

Most ion transporting ATPases have a requirement for Mg$^{2+}$ (24, 25) and although there is no exogenous Mg$^{2+}$ added to the assay mixture, the results from the atomic absorption spectrophotometry measurements have indicated micromolar levels of Mg$^{2+}$ in the assay mixtures. The adipocyte (Ca$^{2+}$ + Mg$^{2+}$)-ATPase (12) was shown to have a requirement for very low levels of Mg$^{2+}$ (< 5 $\mu$M). Therefore, in a test to determine the Mg$^{2+}$ dependence of the ATPase from corn coleoptile plasma membranes, the chelating agent trans-cyclohexane-1,2-diamine-N, N, N', N'-tetraacetic acid (CDTA) was employed to chelate the endogenous Mg$^{2+}$ in the assay mixture, while the Ca$^{2+}$ concentration was varied. The results, also
shown in Figure 7, indicate that at low Ca\(^{2+}\) concentrations, where the chelating agent is an effective buffer of the levels of free Ca\(^{2+}\) and free Mg\(^{2+}\), there is no Ca\(^{2+}\) stimulation of the ATPase, indicating the requirement for Mg\(^{2+}\) by the enzyme. At higher Ca\(^{2+}\) concentrations, perhaps because the CDTA is saturated with Ca\(^{2+}\) ions, allowing the Mg\(^{2+}\) in the mixture to remain free, the ATPase is activated.

Cooperativity of the Binding of Calcium
(Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase

The Hill plot (Figure 8) of the data in Figure 7 indicates that the EGTA-buffered Ca\(^{2+}\) profile has a slope of 1.08, showing no cooperative binding of Ca\(^{2+}\) ions by the ATPase.

Effect of Fluphenazine on the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase Activity

Table 3 shows that in the presence of 50 \(\mu\)M fluphenazine, the Ca\(^{2+}\)-independent ATPase activity is not affected, but the Ca\(^{2+}\)-stimulated activity is inhibited about 60\%. 
DISCUSSION

The presence of a Ca\(^{2+}\)-stimulated ATPase in higher plant membranes was first indicated by experiments with membrane vesicles showing ATP-dependent Ca\(^{2+}\) uptake (13). Since then, the effects of hormones on membrane vesicle Ca\(^{2+}\) uptake have been studied (14) and calmodulin-activation of Ca\(^{2+}\) uptake by plant microsomes has been found (15). Dieter and Marme (16) have also isolated a calmodulin-dependent microsomal ATPase from corn membranes. They demonstrated that the ATPase activity in the microsomes was stimulated about 6% by 550 \(\mu\)M calmodulin and that this stimulation was inhibited by 50 \(\mu\)M fluphenazine. After solubilizing the microsomes with Triton X-100, the calmodulin-stimulated ATPase was chromatographed on a calmodulin-conjugated Sepharose-4B column to remove contaminating proteins. The ATPase obtained after treating the column with EGTA has a definite dependence on added calmodulin. Under optimal conditions, calmodulin produced a 130% stimulation of the ATPase activity. As the authors stated, more work needs to be done with the enzyme to establish the biochemical properties of the ATPase.

In this report, we have studied the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase in corn coleoptile plasma membranes. Our experimental procedures differ significantly in two respects from those of other investigators who have studied plant plasma membrane ATPases: 1) A major problem with detecting and studying a Ca\(^{2+}\)-stimulated ATPase activity in plant plasma membranes is the presence of a (Mg\(^{2+}\) + K\(^{+}\))-ATPase, also present in the plasma membranes. The (Mg\(^{2+}\) + K\(^{+}\))-ATPase is so abundant that it is very diffi-
cul to measure accurately the lower activity of the Ca$^{2+}$-stimulated ATPase. We have altered the standard assay conditions for plant membrane ATPases, by omitting Mg$^{2+}$ and K$^+$ and lowering the ATP concentration, so that the (Mg$^{2+}$ + K$^+$)-ATPase is essentially inactive, while the Ca$^{2+}$-stimulated ATPase is still operating at a rate sufficient to permit reliable measurements of the activity. 2) During the preparation of the plasma membranes from the corn coleoptiles, we have omitted the chelating agent(s) EDTA and EGTA from the homogenization buffer. The inclusion of the chelators in the buffer tends to remove endogenous calmodulin from membranes (26, 27) which in turn greatly enhances the effect of added calmodulin (16, 28). By eliminating EDTA and EGTA from the homogenization buffer, we believe that the membranes and the ATPase will be in a physical state more closely resembling that found in the cell. However, leaving the endogenous calmodulin associated with the membranes tends to decrease or eliminate the effect added calmodulin has on the Ca$^{2+}$-stimulated ATPase activity, making it difficult to show a calmodulin dependence of the enzyme. By treating the tissue homogenate with 5 mM EDTA, Dieter and Marme (15, 16) were able to demonstrate calmodulin stimulation of a Ca$^{2+}$-activated ATPase in the microsomal membranes, but when corn plasma membrane-enriched microsomes are prepared without EDTA present, as in our procedure, we found no stimulation of the ATPase by calmodulin at all (results are not shown). Dieter and Marme (15) also showed that membranes prepared in the absence of EDTA had a very small response to added calmodulin. The results of the experiment testing the effect of fluphenazine on the (Ca$^{2+}$ + Mg$^{2+}$)-ATPase show an inhibition of the ATPase activity (Table 3).
Fluphenazine is a member of a family of phenothiazine compounds which have been shown to inhibit many calmodulin-dependent processes (29, 30). However, the extrapolation of the fluphenazine inhibition to suggest that this enzyme is calmodulin-dependent may not be warranted. There are cases reported (31-33) in which phenothiazines have been shown to inhibit purified enzymes in the absence of calmodulin. Therefore, in spite of a suggestion of the presence of endogenous calmodulin interacting with the \((\text{Ca}^{2+} + \text{Mg}^{2+})-\text{ATPase}\), no conclusive statement can be made until better evidence is obtained upon purifying the enzyme.

Studies of the divalent cation requirements of the ATPase required the determination of the endogenous levels of ions present in the assay mixture. Atomic absorption spectrophotometry indicated the presence of micromolar concentrations of both \(\text{Ca}^{2+}\) and \(\text{Mg}^{2+}\), so that when ion affinity or ion requirement studies were performed, the endogenous ions needed to be accounted for. The determination of the \(K_a\) of \(\text{Ca}^{2+}\) for the membrane-bound ATPase used the chelator EGTA as the ion buffer in the assay mixture. The result, \(K_a = 0.2 \mu\text{M}\ \text{Ca}^{2+}\), is similar to that of calmodulin-dependent ATPases (10, 12, 28). The question of a requirement for \(\text{Mg}^{2+}\) by the ATPase was also addressed at the same time. Utilizing the strong chelating ability of CDTA for both \(\text{Ca}^{2+}\) and \(\text{Mg}^{2+}\) at pH 6.5, we were able to demonstrate that while the EGTA-buffered samples have a high affinity component in a \(\text{Ca}^{2+}\) concentration profile, the CDTA-buffered samples did not have a high affinity \(\text{Ca}^{2+}\)-stimulated ATPase activity. This result indicates a \(\text{Mg}^{2+}\) requirement for the high affinity \(\text{Ca}^{2+}\)-stimulated ATPase activity in the plasma membranes.

The experiments shown in Figure 2 and Figure 7 can be compared
only keeping in mind that there is endogenous Ca\(^{2+}\) and Mg\(^{2+}\) in the membrane preparations, at levels high enough that added Ca\(^{2+}\) and Mg\(^{2+}\) can have little effect on the activity. The marked dependence of the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase on Ca\(^{2+}\) and Mg\(^{2+}\) cannot be seen unless the endogenous metal ions are chelated. Therefore, it cannot be determined from the experiments whether the other divalent cations tested replace Ca\(^{2+}\) or Mg\(^{2+}\) or both on the enzyme.

The effect on the ATPase of a series of inhibitors was tested for comparison with other ATPases. The (Ca\(^{2+}\) + Mg\(^{2+}\)) ATPase is insensitive to oligomycin, sodium azide, sodium molybdate, and sodium vanadate. Oligomycin and sodium azide insensitivity demonstrate that the ATPase activity is not due to contamination of the plasma membrane fraction by mitochondrial material (34). The lack of inhibition by sodium molybdate indicates that the enzyme we have studied is not a Ca\(^{2+}\)-stimulated nonspecific phosphatase (35, 36). Insensitivity to sodium vanadate is difficult to interpret because the \(K_{1/2}\) for vanadate inhibition may be Mg\(^{2+}\) dependent (10, 37). At higher Mg\(^{2+}\) levels needed for vanadate inhibition, the (Mg\(^{2+}\) + K\(^{+}\))-ATPase in the membrane preparation becomes active, masking any effect of vanadate on the (Ca\(^{2+}\) + Mg\(^{2+}\)) ATPase.

The sensitivity of the enzyme to DCCD and DES is similar to that reported for enzymes implicated in the transport of protons (38, 39) and potassium ions (40, 41). Other Ca\(^{2+}\)-stimulated ATPases have apparently not been tested with these inhibitors.

The comparison of the corn plasma membrane Ca\(^{2+}\)-stimulated ATPase to Ca\(^{2+}\)-stimulated ATPases from other sources raises interesting
questions about the extent of homology between plant and animal enzymes. When the corn coleoptile plasma membrane (Ca$^{2+}$ + Mg$^{2+}$)-ATPase is compared to the (Ca$^{2+}$ + Mg$^{2+}$)-ATPase from erythrocyte ghosts and rat liver as well as the rat adipocyte ATPase, there are many similarities in the characteristics of the enzymes. All of the enzymes have sub-micromolar $K_a$ values for Ca$^{2+}$, require Mg$^{2+}$ for activity and have pH optima between pH 6.5-7.5. Also, the enzymes are similar in their responses to a variety of ATPase inhibitors. Subsequent solubilization, purification, and characterization will enable us to better understand the interactions of the (Ca$^{2+}$ + Mg$^{2+}$)-ATPase in the plant cell.
REFERENCES


Table 1. Substrate specificity of the (Ca$^{2+}$ + Mg$^{2+}$)-ATPase

<table>
<thead>
<tr>
<th>100 μM substrate</th>
<th>200 μM EGTA</th>
<th>+ 100 μM Ca$^{2+}$</th>
<th>Ca$^{2+}$ stim.</th>
<th>% of ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>1.31</td>
<td>3.26</td>
<td>1.95</td>
<td>100</td>
</tr>
<tr>
<td>GTP</td>
<td>1.20</td>
<td>2.56</td>
<td>1.36</td>
<td>70</td>
</tr>
<tr>
<td>CTP</td>
<td>0.93</td>
<td>2.02</td>
<td>1.09</td>
<td>56</td>
</tr>
<tr>
<td>UTP</td>
<td>0.89</td>
<td>2.64</td>
<td>1.75</td>
<td>90</td>
</tr>
<tr>
<td>pNPP</td>
<td>1.09</td>
<td>1.54</td>
<td>0.45</td>
<td>23</td>
</tr>
<tr>
<td>Sample</td>
<td>nmoles Pi/h/mg protein</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt; stim. % of control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------------</td>
<td>------------------------</td>
<td>-----------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>+ 100 μM Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt; stim.</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>358</td>
<td>591</td>
<td>233</td>
<td>100</td>
</tr>
<tr>
<td>2 μg/ml oligomycin&lt;sup&gt;a&lt;/sup&gt;</td>
<td>343</td>
<td>584</td>
<td>241</td>
<td>103</td>
</tr>
<tr>
<td>5 μg/ml oligomycin</td>
<td>355</td>
<td>597</td>
<td>242</td>
<td>104</td>
</tr>
<tr>
<td>10 μg/ml oligomycin</td>
<td>357</td>
<td>605</td>
<td>248</td>
<td>106</td>
</tr>
<tr>
<td>0.5 mM Na&lt;sub&gt;2&lt;/sub&gt;MoO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>137</td>
<td>358</td>
<td>221</td>
<td>95</td>
</tr>
<tr>
<td>1.0 mM Na&lt;sub&gt;2&lt;/sub&gt;MoO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>133</td>
<td>343</td>
<td>210</td>
<td>90</td>
</tr>
<tr>
<td>2.0 mM Na&lt;sub&gt;2&lt;/sub&gt;MoO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>164</td>
<td>360</td>
<td>196</td>
<td>84</td>
</tr>
<tr>
<td>10 μM DCCD&lt;sup&gt;a&lt;/sup&gt;</td>
<td>340</td>
<td>529</td>
<td>189</td>
<td>81</td>
</tr>
<tr>
<td>100 μM DCCD&lt;sup&gt;a&lt;/sup&gt;</td>
<td>356</td>
<td>494</td>
<td>138</td>
<td>59</td>
</tr>
<tr>
<td>10 μM DES&lt;sup&gt;a&lt;/sup&gt;</td>
<td>376</td>
<td>535</td>
<td>159</td>
<td>68</td>
</tr>
<tr>
<td>100 μM DES&lt;sup&gt;a&lt;/sup&gt;</td>
<td>400</td>
<td>516</td>
<td>116</td>
<td>50</td>
</tr>
<tr>
<td>0.95% ethanol</td>
<td>320</td>
<td>543</td>
<td>223</td>
<td>96</td>
</tr>
<tr>
<td>0.1 mM NaN&lt;sub&gt;3&lt;/sub&gt;</td>
<td>374</td>
<td>556</td>
<td>182</td>
<td>78</td>
</tr>
<tr>
<td>0.2 mM NaN&lt;sub&gt;3&lt;/sub&gt;</td>
<td>336</td>
<td>579</td>
<td>243</td>
<td>104</td>
</tr>
<tr>
<td>0.5 mM NaN&lt;sub&gt;3&lt;/sub&gt;</td>
<td>358</td>
<td>565</td>
<td>207</td>
<td>89</td>
</tr>
<tr>
<td>1.0 mM NaN&lt;sub&gt;3&lt;/sub&gt;</td>
<td>389</td>
<td>601</td>
<td>212</td>
<td>91</td>
</tr>
</tbody>
</table>

<sup>a</sup>Dissolved in 0.95% ethanol.
Table 3. Effect of fluphenazine on the corn plasma membrane 
(Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase activity

<table>
<thead>
<tr>
<th>Condition</th>
<th>nmoles Pi/h/mg protein</th>
<th>Ca(^{2+}) stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- Ca(^{2+}) (no EGTA)</td>
<td>+ 100 (\mu)M Ca(^{2+})</td>
</tr>
<tr>
<td>- Fluphenazine</td>
<td>723</td>
<td>941</td>
</tr>
<tr>
<td>+ 50 (\mu)M Fluphenazine</td>
<td>720</td>
<td>804</td>
</tr>
</tbody>
</table>
Figure 1. Sucrose density gradient profile. The distribution of the 80,000 x g microsome pellet suspension on a continuous sucrose density gradient. Profiles of the Ca\textsuperscript{2+}-stimulated ATPase activity (●), membrane bands (A\textsubscript{350 nm}) (○) and the sucrose concentrations (Δ) are shown.
Assom (Turbidity)

Ca$^{2+}$ Stimulated μ moles Pi/h/ml fraction

A$_{550\text{nm}}$ (Turbidity)

Fraction

(m/W) Sucrose \%
Figure 2. *Effect of added divalent cations on the plasma membrane ATPase activity.* The ATPase activity was measured in the presence of endogenous levels of 14 μM Mg\(^{2+}\) and 12 μM Ca\(^{2+}\) as increasing amounts of Ca\(^{2+}\) (●), Mg\(^{2+}\) (○), Co\(^{2+}\) (■), Mn\(^{2+}\) (▲), Ni\(^{2+}\) (■) and Zn\(^{2+}\) (▲) were added. The sample without added divalent cation included 200 μM EGTA.
immoles Pi/h/mg protein

μ moles Pi/h/mg protein

Added MgCl₂, µM

[Diagram showing a graph with various lines and markers, indicating the relationship between added MgCl₂ and μ moles Pi/h/mg protein.]
Figure 3. **Effect of increasing ATP concentrations at varying Ca\textsuperscript{2+} concentrations on the plasma membrane ATPase activity.**

The relationship of Ca\textsuperscript{2+}-stimulated ATPase activity to increasing Ca\textsuperscript{2+} levels at various ATP concentrations (■, 0.1 mM; △, 0.2 mM; ○, 0.5 mM; □, 1 mM; ●, 3 mM) is shown.
μ moles Pi/h/mg protein

CaCl₂, mM

0.0 0.1 0.2 0.3 0.4 0.5 1.0 1.5 2.0

0.8 1.0 1.2 1.4 1.6 1.8 2.0

[Graph showing the relationship between CaCl₂ concentration and the uptake of Pi/h/mg protein]
Figure 4. **Time course of Pi production.** The plasma membrane \( \text{Ca}^{2+} \)-stimulated ATPase was assayed in the presence of 10 mM Hepes adjusted to pH 6.5 with imidazole, 100 \( \mu \text{M} \) \( \text{Ca}^{2+} \), and 100 \( \mu \text{M} \) ATP to test for the linearity of product formation with time.
Figure 5. pH profile of the Ca\(^{2+}\)-stimulated ATPase activity. The (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase activity was measured as a function of pH from pH 5.5 to 9.0, using 10 mM Hepes adjusted to the appropriate pH with imidazole.
Figure 6. **Effect of sodium vanadate on the plasma membrane ATPase activity**

Bottom panel: The effect of increasing Na$_3$VO$_4$ on the plasma membrane ATPase activity in the presence (●) and absence (○) of 100 μM Ca$^{2+}$

Top panel: The net Ca$^{2+}$-stimulated ATPase activity (Δ)
Figure 7. Effect of increasing free Ca\(^{2+}\) concentrations on the plasma membrane (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase. The levels of free Ca\(^{2+}\) were buffered in the assay mixture with either EGTA (○) or CDTA (●). Other conditions used are as stated in the Materials and Methods section. The total chelator concentration was 200 μM.
Figure 8. **Hill plot of data from Figure 7.** The data obtained from the free $[\text{Ca}^{2+}]$ profile on the plasma membrane ATPase activity buffered with EGTA was analyzed according to the Hill equation and is shown here. Excluding the three lowest $\text{Ca}^{2+}$ concentrations, a least squares linear regression was used to compute a slope of 1.08.
SECTION III.
PARTIAL PURIFICATION AND CHARACTERIZATION OF A
Ca$^{2+}$-STIMULATED ATPase FROM PLASMA MEMBRANES FROM
*Zea mays* COLEOPTILES

by

STEPHEN G. CARTER and CARL L. TIPTON
A Ca\(^{2+}\)-ATPase activity from the plasma membrane-enriched fraction of corn shoot microsomes has been solubilized, partially purified and characterized. The Ca\(^{2+}\)-stimulated ATPase activity has a molecular weight of 105,000 and a pH optimum at 6.5. The ability of different divalent cations to stimulate the ATPase is in the following order: Ca\(^{2+}\) > Mg\(^{2+}\) > Co\(^{2+}\) > Mn\(^{2+}\) > Ni\(^{2+}\) > Zn\(^{2+}\). The order of substrate preference is: ATP > UTP > CTP > GTP >> pNPP. The Ca\(^{2+}\)-stimulated ATPase activity has two components of Ca\(^{2+}\) affinity. There is a high affinity Ca\(^{2+}\)-ATPase activity with a \(K_a\) = 0.06 \(\mu\)M Ca\(^{2+}\) and a low affinity Ca\(^{2+}\)-ATPase activity with a \(K_a\) = 15 \(\mu\)M Ca\(^{2+}\). The low affinity component requires the presence of monovalent cations to be active. This requirement shows a maximal Ca\(^{2+}\) stimulation response at 125 mM monovalent cation concentration. The Ca\(^{2+}\)-ATPase is completely inhibited by 100 \(\mu\)M fluphenazine, an inhibitor of many calmodulin stimulated processes. Ophiobolin A, a fungal metabolite recently shown to be a potent inhibitor of calmodulin and calmodulin activated processes, also inhibits the Ca\(^{2+}\)-ATPase activity 50% at 25 \(\mu\)M.
INTRODUCTION

The Ca\textsuperscript{2+}-stimulated ATPase from erythrocyte ghost membranes has been well-characterized (1-3) and has been shown to be the enzyme responsible for maintaining an electrochemical gradient of Ca\textsuperscript{2+} ions across the membrane (4). Attempts to study the enzyme in the ghost membrane were hindered by the low amounts of the Ca\textsuperscript{2+}-ATPase present and by the presence of a Mg\textsuperscript{2+}-ATPase (5) which interfered with the determination of many of the properties of the Ca\textsuperscript{2+}-ATPases. The interaction of the Ca\textsuperscript{2+}-ATPase with calmodulin suggested an isolation procedure to be used in the purification of the Ca\textsuperscript{2+}-ATPase. A calmodulin-conjugated Sepharose 4B column was prepared and used for the partial purification of the Ca\textsuperscript{2+}-ATPase from detergent extracted erythrocyte ghost membranes (6). Once the Ca\textsuperscript{2+}-ATPase was purified, many of the properties of the enzyme were studied, in the absence of contaminating proteins (5-7).

This work was followed by Dieter and Marme (8) with the partial purification of a Ca\textsuperscript{2+}-ATPase from corn microsomes, using a calmodulin conjugated Sepharose column to isolate the enzyme. Despite having a relatively pure enzyme, Dieter and Marme did not characterize the biochemical properties of the Ca\textsuperscript{2+}-ATPase. The Ca\textsuperscript{2+}-ATPase activity from a higher plant source has not yet been thoroughly described.

We have previously studied a (Ca\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPase associated with corn coleoptile plasma membranes (Carter and Tipton, unpublished work which can be found in Section II of this dissertation). The properties of this enzyme are compatible with a role in the active transport of
Ca\textsuperscript{2+}. In this paper, we report the solubilization of the ATPase from the plasma membranes, and the partial purification and some characterization of the enzyme in an attempt to establish some of the biochemical properties of the enzyme, and how they may relate to the function of Ca\textsuperscript{2+} transport in higher plants.
Preparation of Corn Coleoptile Plasma Membranes

Corn seedlings (B73H+ x Mo17H+) were grown at 28°C in the dark for 3 days. The etiolated shoots were removed from the seedlings with a razor blade, washed with deionized water and weighed. The shoot segments were added to 0.25 M sucrose, 20 mM imidazole, pH 7.2 with HCl (homogenization buffer) (4 ml/g shoots). The shoots were initially homogenized in a Waring blender, then the crudely homogenized material was transferred to a Polytron homogenizer (Brinkman Corp.) for 30 s at setting '4' followed by 60 s at setting '6'. Polyvinylpyrrolidone (0.25 g/g shoots) was added and thoroughly mixed. The homogenate was filtered through one layer of Miracloth (Calbiochem-Behring Corp., San Diego, CA) to remove the bulk of the fibrous material. The filtrate was then centrifuged at 16,000 x g for 30 minutes in a Sorval GSA rotor. The supernate was centrifuged in a Beckman Ti 45 rotor at 80,000 x g for 30 minutes to obtain the microsome pellet. The pellet was resuspended in the homogenization buffer and layered on a two-step sucrose density gradient (34% over 45%) in 20 mM imidazole-HCl pH 7.2. The gradient was centrifuged in an SW 27 swinging bucket rotor in the Beckman L-8 centrifuge using the slow acceleration mode for 2 h at 112,840 x g. The plasma membrane-enriched fraction from the microsomes was collected with a Pasteur pipet at the 34%/45% interface.
Extraction of the \( \text{Ca}^{2+} \)-ATPase from the Plasma Membranes

The plasma membrane fraction was dialyzed for 12 h against the homogenization buffer at 4°C, then extracted with 1% Zwittergent 3,12 (Calbiochem-Behring Corp.) in 1 M KCl. The undissolved membrane material was removed by centrifuging at 80,000 \( \times g \) for 30 minutes. The supernatant was removed and dialyzed for 6 h against the homogenization buffer followed by 12 h against 0.25 M NaClO\(_4\), 20 mM imidazole pH 8.0 with HCl (buffer B).

Hexyl Agarose Column Chromatography

The dialysis retentate was applied to a 1.6 x 30 cm column of hexyl agarose (Miles-Yeda Laboratories) previously equilibrated with buffer B. The column was eluted with 60 ml of buffer B followed by 60 ml of 2 M NaClO\(_4\), 20 mM imidazole-HCl, pH 8.0 (buffer C). The column fractions were assayed for \( \text{Ca}^{2+} \)-ATPase and \( \text{(Mg}^{2+} + \text{K}^+ \)-ATPase, and \( A_{280\text{nm}} \) and the conductivity were measured.

CM-52 Cellulose Column Chromatography

The \( \text{Ca}^{2+} \)-ATPase eluted from the hexyl agarose column with the high salt buffer was dialyzed against 1 M NaCl, 20 mM imidazole-HCl, pH 8.0. The dialysis retentate was concentrated with Aquacide II-A (Calbiochem-Behring Corp.) and then, immediately prior to application to a 1.6 x 15 cm CM-Cellulose column, diluted with deionized water to 0.1 M NaCl concentration. The enzyme mixture was applied to the column,
washed with 50 ml 0.1 M NaCl, 10 mM imidazole-HCl, pH 8.0, followed by
1 M NaCl, 20 mM imidazole-HCl, pH 8.0.

Sephacryl S-200 Column Chromatography

The Ca\(^{2+}\)-ATPase obtained from the high salt elution of the CM-
Cellulose column was concentrated with Aquacide II-A to approximately
10 ml and applied to a Sephacryl S-200 column (2.5 x 80 cm) which was
equilibrated with 0.25 M NaCl, 20 mM imidazole-HCl, pH 8.0. The
column was eluted with the same buffer at a flow rate of 0.77 ml per
minute.

Sephadex G-100 Column Chromatography

The high salt fraction of Ca\(^{2+}\)-ATPase from the CM-Cellulose column
was concentrated with Aquacide II-A to approximately 5 ml. The
concentrated ATPase was then applied to a Sephadex G-100 column
(2.6 x 86 cm) equilibrated with 1 M NaCl, 20 mM imidazole-HCl, pH
8.0. The ATPase was eluted from the column at a flow rate of 1 ml/
minute.

ATPase Assays

Enzymes were assayed under the following conditions unless other­
wise stated: (Mg\(^{2+}\) + K\(^{+}\))-ATPase = 33 mM MES-Tris, pH 6.0, 50 mM KCl,
1.5 mM MgCl\(_2\), 3 mM ATP (9); Ca\(^{2+}\)-ATPase = 10 mM Hepes-imidazole, pH 6.5,
with and without 100 \mu M \text{CaCl}_2, 125 \text{mM NaCl or KCl}, 100 \mu M \text{ATP}. Inorganic phosphate was estimated as described earlier (10).

Protein Determinations

A TCA precipitation variation of the procedure of Lowry et al. (11) was used.

Determination of Divalent Metal Ion Concentrations

The levels of Mg$^{2+}$ and Ca$^{2+}$ in the enzyme assay mixtures were estimated by atomic absorption spectrophotometry with a Varian Techtron Model 1200 atomic absorption spectrophotometer, with a combination Ca$^{2+}$/Mg$^{2+}$/Ni$^{2+}$ lamp source.

Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis

A Laemmli (12) gel system (3% stacking and 8% separating gels) was used for electrophoretic analysis of the protein solutions under denaturing conditions.
RESULTS

Purification of the Ca\textsuperscript{2+}-ATPase from Corn Shoot Plasma Membranes

The plasma membrane enriched fraction from the corn shoot microsomes was isolated on a two-step sucrose density gradient, extracted with detergent and KCl, dialyzed and applied to a hexyl agarose column. Figure 1 shows that the hexyl agarose column resolves two peaks of Ca\textsuperscript{2+}-ATPase activity from the crude plasma membrane extract. The Ca\textsuperscript{2+}-ATPase eluted from the column with the low salt buffer was of low specific activity and it coincided with the majority of the (Mg\textsuperscript{2+} + K\textsuperscript{+})-ATPase activity from the extract (data not shown). The other Ca\textsuperscript{2+}-ATPase fraction, which remained on the column during the low salt wash, was eluted from the column with high salt. This Ca\textsuperscript{2+}-ATPase was higher in specific activity and there was very little (Mg\textsuperscript{2+} + K\textsuperscript{+})-ATPase present. The 'high salt' hexyl agarose Ca\textsuperscript{2+}-ATPase fraction was concentrated with Aquacide II-A for further purification and characterization.

CM-52 Cellulose Column Chromatography

The concentrated Ca\textsuperscript{2+}-ATPase from the high salt hexyl agarose fraction was dialyzed against 1 M NaCl, 20 mM imidazole-HCl, pH 8.0 for 24 h at 4°C. The dialysis retentate was diluted to 0.1 M NaCl with deionized water immediately prior to application to the CM-Cellulose, then applied to the gel, washed with 60 ml 0.1 M NaCl buffer followed with 60 ml 1 M NaCl buffer. The enzyme loses activity in salt solutions less than 0.25 M so these steps must be carried out rapidly. A low specific activity K\textsuperscript{+}-ATPase elutes with the majority of the protein.
in the low salt wash of the column. The Ca^{2+}-ATPase is eluted from the column with the high salt buffer (Figure 2). The high salt CM-Cellulose Ca^{2+}-ATPase is the partially purified fraction with which most of the characterization of the Ca^{2+}-stimulated ATPase activity was performed.

The Ca^{2+}-stimulated ATPase from corn shoot plasma membranes has been purified 25-fold from the plasma membrane fraction to the high salt fraction of the CM-Cellulose column (Table 1).

Sephacryl S-200 Column Chromatography

The Ca^{2+}-ATPase obtained from the high salt wash of the CM-Cellulose column was concentrated in preparation for a molecular weight estimation by gel filtration on a Sephacryl S-200 column. The ATPase was placed on the column and eluted with 0.25 M NaCl, 20 mM imidazole-HCl, pH 8.0. The resulting profile, shown in Figure 3 corresponds to a $M_r$ of 8,500 when compared with molecular weight standards.

Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis (SDS/PAGE)

The enzyme eluted from the Sephacryl S-200 column was analyzed by SDS/PAGE. As shown in Figure 4, there are at least 5 protein bands visible on the gel, with mobilities corresponding to molecular weights of 68,000, 60,000, 28,000, 25,500, and 24,500.
Sephadex G-100 Column Chromatography

The Ca\(^{2+}\)-ATPase from the high salt wash of the CM-Cellulose column was concentrated and prepared for chromatography on a Sephadex G-100 column. The elution profile obtained for the Ca\(^{2+}\)-ATPase activity on the calibrated Sephadex G-100 column is shown in Figure 5. This elution position corresponds to a molecular weight for the Ca\(^{2+}\)-ATPase of 105,000 +/- 6000 daltons.

Substrate Specificity for the CM-Cellulose Treated Ca\(^{2+}\)-ATPase

The Ca\(^{2+}\)-ATPase activity eluted from the CM-Cellulose column was tested to determine what substrate was best for maximal Ca\(^{2+}\) stimulation to occur. Table 2 shows that the order of substrate preference is ATP > UTP > CTP > GTP > pNPP.

Effect of MgCl\(_2\) and Calmodulin on the CM-Cellulose Treated Ca\(^{2+}\)-ATPase Activity

Table 3 shows the effect of 1 mM MgCl\(_2\) and/or 2.5 units of bovine brain calmodulin on the Ca\(^{2+}\)-ATPase eluted from the CM-Cellulose column. The addition of MgCl\(_2\) suppresses the basal ATPase activity ~40% (in the presence of EGTA), but the addition of 1 mM Ca\(^{2+}\) to the sample yields the same Ca\(^{2+}\) stimulation as the control samples. The addition of 2.5 units of calmodulin to the assay mixture results in a 32% stimulation of the Ca\(^{2+}\) stimulation. The result of combining the two substances, again is a reduction in the basal ATPase activity, but the
additional Ca\(^{2+}\) stimulation seen with the addition of only calmodulin is abolished. The presence of MgCl\(_2\) with the calmodulin has essentially the same Ca\(^{2+}\) stimulation as the control.

pH Profile of the Hexyl Agarose 'High Salt' Ca\(^{2+}\)-ATPase

Figure 6 shows that the pH profile of the Ca\(^{2+}\)-stimulated ATPase activity has a steep peak of activity with a maximum at pH 6.5.

Effect of Different Divalent Cations at Varying Concentrations on the Ca\(^{2+}\)-Stimulated ATPase Activity

Figure 7 shows the effect of increasing concentrations of divalent cations on the Ca\(^{2+}\)-ATPase activity. Ca\(^{2+}\) is the best stimulator of the ATPase at all concentrations tested, although at 100 \(\mu\)M MgCl\(_2\), Mn\(^{2+}\) and Co\(^{2+}\) have essentially the same effect as Ca\(^{2+}\). Atomic absorption spectrophotometry of the enzyme assay mixture without added metal salts indicated the presence of 2.8 \(\mu\)M Mg\(^{2+}\) and 7.0 \(\mu\)M Ca\(^{2+}\). The presence of this much Ca\(^{2+}\) and Mg\(^{2+}\) may be enough to interfere with the true effects of the other divalent cations on the ATPase.

Requirement for Monovalent Cations by the Ca\(^{2+}\)-ATPase

The Ca\(^{2+}\)-ATPase activity has a requirement for monovalent cations (Figure 8). The optimum concentration of Na\(^+\) and K\(^+\) is approximately 125 mM. Four different ions were tested for their ability to stimulate
the Ca\(^{2+}\)-ATPase. The order of the activating ability is: K\(^+\) > Li\(^+\) > NH\(_4\)^+ > Na\(^+\).

\(\text{Ca}^{2+}\) Affinity Constant Determination

The apparent affinity constant for Ca\(^{2+}\) of the enzyme eluted with high salt from the hexyl agarose column was determined. The Lineweaver-Burk plot (Figure 9) shows two binding events occurring with this enzyme sample and different Ca\(^{2+}\) concentrations. The enzyme mixture appears to have a high affinity component with a \(K_a\) of 0.06 \(\mu\)M Ca\(^{2+}\) and a low affinity component with a \(K_a\) of 15 \(\mu\)M Ca\(^{2+}\). The effect of monovalent cations on the CM-Cellulose high salt ATPase kinetics is shown in Figure 10. Monovalent cations in the assay mixture increases the \(V_{\text{max}}\) of the high affinity Ca\(^{2+}\)-ATPase reaction without affecting the affinity for Ca\(^{2+}\). The presence of monovalent cations not only increases the \(V_{\text{max}}\) of the high affinity Ca\(^{2+}\)-ATPase activity but are also required for the low affinity component to be active.

Effect of Fluphenazine and Ophiobolin A on the Ca\(^{2+}\)-ATPase Activity

The effect of fluphenazine, an inhibitor of many calmodulin-activated processes, on the Ca\(^{2+}\)-stimulated ATPase activity is shown in Figure 11. The Ca\(^{2+}\)-ATPase activity is completely inhibited by fluphenazine at 100 \(\mu\)M and is 50% inhibited at approximately 50 \(\mu\)M fluphenazine.

Ophiobolin A, a metabolite produced by \textit{Helminthosporium} fungi, has been shown to be an inhibitor of calmodulin and processes affected
by calmodulin (unpublished results from P.-C. Leung and C. Tipton, Iowa State University, and W. Taylor and J. Wang, University of Winnipeg). The addition of ophiobolin A to the Ca$^{2+}$-ATPase inhibits the Ca$^{2+}$-stimulated activity 50% at about 25 μM ophiobolin A (Figure 12).
In this paper, we report the partial purification of a $\text{Ca}^{2+}$-ATPase activity from the plasma membranes of corn coleoptiles. The ATPase appears to have properties which suggest a role in the active transport of $\text{Ca}^{2+}$ ions across the plasma membrane of the corn cell. The determination of the $\text{Ca}^{2+}$ affinity constant(s) for the enzyme indicates the presence of two classes of binding sites present in the high salt hexyl agarose ATPase and in the more purified high salt CM-Cellulose enzyme mixture. Possible reasons for this observation are: 1) there is a single $\text{Ca}^{2+}$-ATPase which has two binding sites for $\text{Ca}^{2+}$ on the same protein molecule, but with different affinities for the ion, or 2) there are two different $\text{Ca}^{2+}$-ATPases in the partially purified fractions, each having a different affinity for $\text{Ca}^{2+}$. At this point in the purification and characterization of the ATPase activity, it is impossible to state which possibility is the real case. The enzyme becomes increasingly unstable after treatment on the CM-Cellulose column; therefore, more work on stabilizing the enzyme must be done before further purification steps can be carried out and the subsequent kinetic studies are performed.

The ATPase has an interesting requirement for monovalent cations to be present for the low affinity component of the $\text{Ca}^{2+}$-ATPase activity to be active. The requirement has a sharp concentration optimum but a low specificity for the species of cation, since all four cations tested ($\text{K}^+$, $\text{Na}^+$, $\text{NH}_4^+$, $\text{Li}^+$) were able to satisfy the ATPase requirement, but to varying degrees. It is possible that a role the monovalent cations
may play with the Ca$^{2+}$-ATPase is as a cotransported ion along with the Ca$^{2+}$ ions, but such a statement at this point is purely speculative.

The assignment of a molecular weight to the ATPase was not as straightforward a task as is usually expected. Our first attempt to establish a molecular weight employed a Sephacryl S-200 gel column. When the ATPase was applied to and eluted from the column, the Ca$^{2+}$-ATPase activity eluted at a position corresponding to a $M_r = 8,500$. This unusually low molecular weight estimate was suspect since most of the other ion-transporting ATPases which have been solubilized and partially purified have molecular weights in the vicinity of 100,000 daltons. Sephacryl S-200 gels have been shown to nonspecifically absorb some proteins (13, 14). The authors of these papers describe conditions to be used with the Sephacryl gel to avoid this problem, and these are the conditions we used with our ATPase. Despite our precautions, we suspected adsorption of the ATPase occurred with the Sephacryl gel. Additional evidence that this was the case was obtained when SDS/PAGE was performed on the Sephacryl S-200 column treated Ca$^{2+}$-ATPase fraction. A series of protein bands ranging from 68,000 to 24,500 daltons were observed. The presence of the higher molecular weight protein bands from a gel filtration fraction supposedly corresponding to a molecular weight of 8,500 confirms that there are some unexpected interactions occurring between the protein mixture and the Sephacryl gel. To avoid this, we have used a different gel support to determine the molecular weight of the Ca$^{2+}$-ATPase. A Sephadex G-100 column was used and this time an estimate of $M_r = 105,000 +/- 6000$ was obtained.

Figure 5 shows the elution profile of the ATPase activity from
the Sephadex G-100 column, assayed under conditions which would activate both the Ca$^{2+}$-ATPase and the K$^+$-ATPase present in the hexyl agarose high salt ATPase pool. Peak A corresponds to a molecular weight of 105,000 and when assayed was shown to contain a Ca$^{2+}$-stimulated ATPase activity. Peak B eluted at a volume corresponding to a molecular weight of 30,000 and upon assay, was found to be a K$^+$-ATPase.

As mentioned in our report of the plasma membrane-bound (Ca$^{2+}$ + Mg$^{2+}$)-ATPase from corn coleoptiles in Section II of this dissertation, the method we use to prepare plasma membranes (without EGTA or EDTA), minimizes the loss of any endogenous calmodulin associated with the membranes. The result of this process is that there is little, if any, effect on the ATPase by added calmodulin, making it difficult to show a calmodulin dependence or stimulation. It seems unlikely that endogenous calmodulin would remain associated with the Ca$^{2+}$-ATPase after solubilization from the membranes and two column chromatography treatments; however, the question of a calmodulin requirement for the ATPase still remains unanswered. Addition of 2.5 units of bovine brain calmodulin to the ATPase has only a minimal stimulating effect on the partially purified Ca$^{2+}$-ATPase activity (Table 3). Fluphenazine, an inhibitor of many calmodulin stimulated processes, is a very effective inhibitor of the Ca$^{2+}$-ATPase (Figure 11). The inhibition of fluphenazine does not directly indicate the presence of calmodulin since the fluphenazine may be interacting with calmodulin or it may be interacting with the ATPase directly causing the inhibition of the ATPase activity (15-17). We also tested the effect of ophiobolin A on the Ca$^{2+}$-ATPase activity. Ophiobolin A is a metabolite produced by the Helminthosporium fungi and
has been found to be a very potent inhibitor of calmodulin and calmodulin-stimulated processes. Ophiobolin A also strongly inhibited the Ca\(^{2+}\)-ATPase activity, with a 50% inhibition at 25 μM. These experiments indicate that calmodulin is associated with the Ca\(^{2+}\)-ATPase, but until we can remove the calmodulin from the system and eliminate the Ca\(^{2+}\)-stimulated ATPase activity, then add back calmodulin and restore the Ca\(^{2+}\)-stimulated activity, we cannot make a definite statement about whether calmodulin is present.

In an attempt to draw an analogy between the corn plasma membrane Ca\(^{2+}\)-ATPase and the erythrocyte ghost Ca\(^{2+}\)-ATPase, we tried to stabilize and/or enhance the Ca\(^{2+}\) activation of the enzyme by adding phospholipids to the assay mixture. Some workers with the erythrocyte Ca\(^{2+}\)-ATPase used phosphatidylcholine (5) and phosphatidylserine (7) in the solubilization procedure and in the assay mixtures to stabilize the ATPase and to mimic the effect of calmodulin. The corn plasma membrane solubilized Ca\(^{2+}\)-ATPase did not respond to the phosphatidylcholine or the phosphatidylserine (results not shown). Despite negative results with the effects of these two phospholipids on the ATPase, experiments along this line should continue with other phospholipids since a positive result would be very useful when reconstitution experiments are performed with the enzyme.
REFERENCES

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (mg)</th>
<th>Total activity (μmoles Pi/h)</th>
<th>% recovery</th>
<th>Specific activity (μmoles Pi/h/mg)</th>
<th>Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma membrane enriched microsome fraction</td>
<td>4.04</td>
<td>9.2</td>
<td>100</td>
<td>2.3</td>
<td>1</td>
</tr>
<tr>
<td>High salt hexyl agarose ATPase</td>
<td>2.20</td>
<td>14.7</td>
<td>159</td>
<td>6.7</td>
<td>3</td>
</tr>
<tr>
<td>High salt CM-Cellulose ATPase</td>
<td>0.11</td>
<td>6.3</td>
<td>69</td>
<td>56.4</td>
<td>25</td>
</tr>
</tbody>
</table>
Table 2. Substrate specificity of the CM-Cellulose treated $\text{Ca}^{2+}$-ATPase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$\mu$moles Pi/h/mg protein</th>
<th>200 $\mu$M EGTA</th>
<th>1 mM $\text{Ca}^{2+}$</th>
<th>$\text{Ca}^{2+}$ stim.</th>
<th>% of ATP $\text{Ca}^{2+}$ stim.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>79.1</td>
<td>131.0</td>
<td>51.9</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>GTP</td>
<td>33.0</td>
<td>50.8</td>
<td>17.8</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>CTP</td>
<td>74.4</td>
<td>115.5</td>
<td>41.1</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>UTP</td>
<td>66.9</td>
<td>110.9</td>
<td>44.0</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>pNPP</td>
<td>28.3</td>
<td>29.4</td>
<td>1.1</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Effect of MgCl$_2$ and calmodulin on the CM-Cellulose treated $\text{Ca}^{2+}$-ATPase activity

<table>
<thead>
<tr>
<th>Additions</th>
<th>$\mu$moles Pi/h/mg protein</th>
<th>200 $\mu$M EGTA</th>
<th>1 mM $\text{Ca}^{2+}$</th>
<th>$\text{Ca}^{2+}$ stim.</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additions</td>
<td>7.7</td>
<td>15.8</td>
<td>8.1</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>1 mM MgCl$_2$</td>
<td>4.3</td>
<td>12.3</td>
<td>8.0</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>2.5 units calmodulin</td>
<td>6.4</td>
<td>17.1</td>
<td>10.7</td>
<td>132</td>
<td></td>
</tr>
<tr>
<td>1 mM MgCl$_2$ +</td>
<td>4.6</td>
<td>12.1</td>
<td>7.5</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>2.5 units calmodulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. Hexyl agarose column chromatography of Zwittergent-extracted corn shoot plasma membranes. The profiles shown are: ●, Ca^{2+}-ATPase activity; ○, A_{280nm}; and △, conductivity (µHΩS). The assay conditions used for the Ca^{2+}-ATPase profile are 10 mM Hepes-imidazole, pH 6.5, 100 µM CaCl₂, 100 mM NaClO₄, 100 µM ATP.
Figure 2. CM-Cellulose column chromatography of hexyl agarose treated Ca$^{2+}$-ATPase, eluted with high salt buffer. The profiles shown are: *, Ca$^{2+}$-ATPase activity (A$_{625\text{nm}}$); ○, protein; Δ, conductivity (μMhos). The assay conditions used for the Ca$^{2+}$-ATPase profile are 10 mM Hepes-imidazole, pH 6.5, 100 μM CaCl$_2$, 100 mM NaCl, 100 μM ATP
Figure 3. Sephacryl S-200 column chromatography of Ca$^{2+}$-ATPase activity after being chromatographed on the CM-Cellulose column. The Ca$^{2+}$-ATPase activity was measured as described in the Materials and Methods section and is expressed as A$_{625}$nm. The ATPase eluted from the column in the buffer 0.25 M NaCl, 20 mM imidazole-HCl, pH 8.0 at a flow rate of 0.77 ml/min.
Figure 4. The gel electrophoresis of Sephacryl S-200 treated Ca$^{2+}$-ATPase. The Ca$^{2+}$-ATPase obtained from the Sephacryl S-200 column was precipitated with trichloroacetic acid, washed with cold acetone and electrophoresed at a constant current of 20 mA. The gel was constructed according to Laemmli (12) with a stacking gel of 3% and an 8% separating gel in 0.1% SDS.
Figure 5. Sephadex G-100 column chromatography of hexyl agarose treated Ca\textsuperscript{2+}-ATPase activity. The Ca\textsuperscript{2+}-ATPase activity obtained from the high salt wash of the hexyl agarose column was applied to the Sephadex G-100 column and eluted with 1 M NaCl, 20 mM imidazole-HCl, pH 8.0 at a flow rate of 1 ml/min. The assay conditions used for this profile are: 10 mM Hepes-imidazole, pH 6.5, 100 μM CaCl\textsubscript{2}, 100 mM NaCl, 100 μM ATP.
Figure 6. **pH profile of hexyl agarose high salt Ca\textsuperscript{2+}-ATPase activity.** The Ca\textsuperscript{2+}-stimulated ATPase activity was measured at a variety of pH values in 10 mM Hepes adjusted to the appropriate pH with imidazole.
Figure 7. Effect of increasing concentrations of different divalent cations on the total ATPase activity from the CM-Cellulose column. Two hundred µM EGTA was used as the sample without any divalent cations present. However, the other points shown in the graph have endogenous levels of 2.8 µM Mg\textsuperscript{2+} and 7.0 µM Ca\textsuperscript{2+} present in the assay mixtures in addition to the added amounts of the different cations. The effect of added Ca\textsuperscript{2+} (●), Mg\textsuperscript{2+} (○), Co\textsuperscript{2+} (□), Mn\textsuperscript{2+} (△), Ni\textsuperscript{2+} (■), and Zn\textsuperscript{2+} (▼) on the CM-Cellulose treated Ca\textsuperscript{2+}-ATPase are shown.
Figure 8. Effect of increasing concentrations of monovalent cations on the Ca$^{2+}$-ATPase from the CM-Cellulose column. The Ca$^{2+}$-ATPase activity is measured in the presence of: NaCl (●), KCl (○), NH$_4$Cl (□), and LiCl (△)
Figure 9. Lineweaver-Burk plot of the effect of increasing levels of Ca\(^{2+}\) on the hexyl agarose 'high salt' Ca\(^{2+}\)-ATPase. The enzyme was assayed according to the conditions described in the Materials and Methods section, except that 100 mM NaClO\(_4\) was present in the assay mixture.
Figure 10. **Lineweaver-Burk plot of the CM-Cellulose treated Ca^{2+}-stimulated ATPase activity in the presence (●) and absence (○) of 125 mM KCl**
Figure 11. Effect of increasing concentrations of fluphenazine on Ca\textsuperscript{2+}-ATPase activity. Increasing amounts of fluphenazine were added to the CM-Cellulose treated Ca\textsuperscript{2+}-ATPase preparation. The Ca\textsuperscript{2+}-stimulated ATPase activity was measured as a function of increasing fluphenazine after a 30-minute incubation at 25\textdegree C.
Figure 12. **The effect of increasing levels of ophiobolin A on Ca\(^{2+}\)-ATPase activity.** The Ca\(^{2+}\)-stimulated ATPase activity was assayed according to the conditions described in the Materials and Methods section after a 30-minute incubation at 25°C with increasing concentrations of ophiobolin A.
GENERAL SUMMARY OF THE DISSERTATION

The 5'-nucleotidase from corn coleoptile microsomes has been purified 125-fold to apparent homogeneity. The enzyme has a native molecular weight of 49,000 daltons according to gel filtration and denaturing gel electrophoresis has shown that two subunits of 24,500 and 25,500 daltons comprise the quaternary structure of the 5'-nucleotidase. The enzyme was shown to be a glycoprotein, containing 40% as much carbohydrate as protein. The 5'-nucleotidase hydrolyses nucleoside monophosphates, with a slight preference for purine nucleotides over pyrimidine nucleotides. Adenosine, a product of the reaction non-competitively inhibits the 5'-nucleotidase ($K_i < 57 \mu M$) while cAMP has been shown to be a competitive inhibitor of the enzyme with a $K_i = 5.2 \mu M$. The possible in vivo function for the 5'nucleotidase in the regulation of the nucleotide pool size or in the cyclic nucleotide metabolism of the cell has been discussed.

The characterization of the (Ca$^{2+} +$ Mg$^{2+}$)-ATPase from the plasma membrane enriched fraction of the microsomes of corn coleoptiles shows a pH optimum at 6.5. The enzyme substrate specificity is in the order of: ATP > UTP > GTP > CTP >> pNPP and the ability of different divalent cations to stimulate the enzyme is as follows: Ca$^{2+}$ > Mg$^{2+}$ > Co$^{2+}$ > Mn$^{2+}$ > Ni$^{2+}$ > Zn$^{2+}$. The (Ca$^{2+} +$ Mg$^{2+}$)-ATPase is not affected by oligomycin, sodium azide, sodium molybdate, or sodium vanadate but is inhibited by DES and DCCD. The Ca$^{2+}$ affinity constant determination for the membrane bound ATPase indicated a $K_a = 0.2 \mu M$ Ca$^{2+}$ and a definite dependence on Mg$^{2+}$. Fluphenazine, an inhibitor of many calmodulin
stimulated processes inhibited the Ca\textsuperscript{2+}-stimulated ATPase activity 60% at 50 \(\mu\)M, but the addition of calmodulin to the plasma membrane fraction did not activate the enzyme further.

The solubilization of the Ca\textsuperscript{2+}-ATPase activity from the plasma membrane-enriched microsome fraction of corn coleoptiles and subsequent purification procedures resulted in a 25-fold purification from the plasma membranes. The molecular weight of the ATPase is 105,000 +/- 6000. A kinetic analysis of the ATPase activity as a function of Ca\textsuperscript{2+} concentration showed the presence of two Ca\textsuperscript{2+} affinity binding activities. There is a high affinity Ca\textsuperscript{2+}-ATPase activity with a \(K_a = 0.06 \mu\)M Ca\textsuperscript{2+} and a low affinity Ca\textsuperscript{2+}-ATPase activity with a \(K_a = 15 \mu\)M Ca\textsuperscript{2+}. The low affinity component requires the presence of 125 mM monovalent cations for maximal activity. The addition of calmodulin to the enzyme mixture does not enhance the Ca\textsuperscript{2+}-stimulated ATPase activity to a significant extent. However, the addition of 100 \(\mu\)M fluphenazine completely inhibits the Ca\textsuperscript{2+}-ATPase activity and the presence of ophiobolin A, a fungal metabolite recently shown to be a potent inhibitor of calmodulin activated processes, inhibits the Ca\textsuperscript{2+}-ATPase activity 50% at 25 \(\mu\)M. The pH profile, which indicates an optimum at 6.5, along with the sub-micromolar affinity constant for Ca\textsuperscript{2+} activation of the enzyme, the substrate specificity and the cation specificity are all properties which are compatible with an enzyme which could be responsible for the active transport of Ca\textsuperscript{2+} ions across the plasma membrane.
ACKNOWLEDGMENTS

I would like to express my thanks to my major professor, Dr. Carl L. Tipton, for his support, guidance and friendship during the course of my graduate studies.

Also, I am grateful to Drs. Alan Atherly, G. Stanley Cox, Merlin Kaeberle, Darryll Outka, and James Thomas for serving on my committee.

Thanks to my present and former student colleagues and friends, Daniel Karl, Michael Davis, Pak Leung, Alan Sharp, Thomas Girard, Paul Braun, and Richard Tamura for some stimulating discussions and necessary diversions. Also, thanks to Simon Goldbard for help in developing and analyzing antibodies and for some great squash.

Finally, no words can properly acknowledge or express my appreciation and thanks to my wife, Pat, and my son, Andrew, whose love and understanding were always there. Thank you.