Partial Purification and Characterization of a Ca\(^{2+}\)-Dependent Protein Kinase from the Green Alga, *Dunaliella salina*

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**ABSTRACT**

A calcium-dependent protein kinase was partially purified and characterized from the green alga *Dunaliella salina*. The enzyme was activated at free Ca\(^{2+}\) concentrations above 10\(^{-5}\) molar, and half-maximal activation was at about 3 \(\times\) 10\(^{-5}\) molar. The optimum pH for its Ca\(^{2+}\)-dependent activity was 7.5. The addition of various phospholipids and diolene had no effects on enzyme activity and did not alter the sensitivity of the enzyme toward Ca\(^{2+}\). The enzyme was inhibited by calmodulin antagonists, N-(6-aminohexyl)-1-naphthalene sulfonamide and N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide in a dose-dependent manner while the protein kinase C inhibitor, sphingosine, had little effect on enzyme activity up to 800 micromolar. Immunodassay showed some calmodulin was present in the kinase preparations. However, it is unlikely the kinase was calmodulin regulated, since it still showed stimulation by Ca\(^{2+}\) in gel assays after being electrophoretically separated from calmodulin by two different methods. This gel method of detection of the enzyme indicated that a protein band with an apparent molecular weight of 40,000 showed protein kinase activity at each one of the several steps in the purification procedure. Gel assay analysis also showed that after native gel isoelectric focusing the partially purified kinase preparations had two bands with calcium-dependent activity, at isoelectric points 6.7 and 7.1. By molecular weight, by isoelectric point, and by a comparative immunodassay, the *Dunaliella* kinase appears to differ from at least some of the calcium-dependent, but calmodulin and phospholipid independent kinases described from higher plants.

Ca\(^{2+}\) ions play key roles in signal transduction and in the regulation of many other cellular processes in plant cells (13, 28). Among the many different cellular processes regulated by Ca\(^{2+}\), the phosphorylation and dephosphorylation of proteins are thought to be particularly important because they can have potent effects on the activity of key metabolic enzymes (26).

Several Ca\(^{2+}\)-dependent protein kinases have been identified in higher plants. Among them some are calmodulin-independent (3, 4, 24, 29) and Ca\(^{2+}\)/phospholipid-dependent protein kinases (8, 20, 30). Other CDPKs, which are calmodulindependent, have been partially purified and characterized from soybean, alfalfa, and apple fruit (1, 5, 12).

Since Ca\(^{2+}\) is also involved in many cellular functions in lower plants as it is in higher plants (13), it is reasonable to believe that there must be Ca\(^{2+}\) regulated protein kinases present in these organisms. However, there is little information available on this topic. Recently, Roberts (27) detected a calcium-activated protein kinase in *Mougeotia* and showed it to be calmodulin- and phospholipid-independent. Here we report the detection of a calcium-activated protein kinase in another green alga, *Dunaliella*, and we show that it, too, appears to be calmodulin- and phospholipid-independent. In addition, we provide information on its partial purification, its mol wt, its pl, and the pH optimum of its activity. Our results suggest that this enzyme differs from other CDPKs in many respects even though it belongs to the same general class of protein kinases.

**MATERIALS AND METHODS**

**Chemicals**

\(^{[\gamma-32P]}\)ATP was from New England Nuclear (specific activity 3000 Ci/mmole). W\(_5\), W\(_7\), calf thymus histone H1 type III-S, bovine brain PS, DO, bovine brain sphingosine, leupeptin, soybean trypsin inhibitor, and spinach calmodulin were from Sigma. Phenyl-Sepharose CL-4B was from Pharmacia. Hydroxylapatite was obtained from Calbiochem.

**Purification of the Protein Kinase**

Cultures of *Dunaliella salina* (UTEX 1644) were grown as previously described (21). Cells were collected by centrifugation at 4000 rpm in a Beckman JA-10 rotor for 5 min and resuspended in about 10 volumes of extraction buffer containing 0.4 M mannitol, 25 mM Tris-Cl (pH 7.5), 2 mM EDTA, 10 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 0.2 mM PMSF. The cell suspension was placed in a pre-chilled Parr cell disruption bomb. After being equilibrated for 12 min at 120 lbs/in\(^2\) N\(_2\) gas, the suspension was released. The broken cell homogenate was centrifuged at 39,100g for 40 min. Ammonium sulfate was added to the supernatant to 50% saturation. After standing for 1 h, the precipitate was centrifuged at 39,100g for 50 min. The pellets were dissolved in about 20 mL of 25 mM Tris-Cl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 0.2 mM PMSF, and clarified by centrifugation at 13,600g in a microcentrifuge for 10 min.

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2. Abbreviations: CDPK, Ca\(^{2+}\)-dependent protein kinase; W\(_5\), N-(6-aminohexyl)-1-naphthalene sulfonamide; W\(_7\), N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide; PS, phosphatidylserine; DO, diolene; PKC, protein kinase C; IEF, isoelectric focusing; pl, isoelectric point.
To the supernatant, CaCl$_2$ and MgCl$_2$ were added to a final concentration of 4 and 2 mM, respectively, and leupeptin and soybean trypsin inhibitor were added to 40 μg/mL each. After stirring for 30 min, the sample was loaded onto a Phenyl-Sepharose column (1.5 x 7 cm) preequilibrated with a buffer containing 25 mM Tris-Cl (pH 7.5), 0.2 mM CaCl$_2$, 0.1% (v/v) 2-mercaptoethanol, 0.2 mM PMSF, 40 μg/mL leupeptin, and 40 μg/mL soybean trypsin inhibitor. The column was washed with 8 bed volumes of the same buffer followed by the above buffer plus 1 M NaCl, then eluted with equilibration buffer containing 2 mM EGTA instead of 0.2 mM CaCl$_2$. Two mL fractions were collected and every other fraction was assayed for enzyme activity. Active fractions were pooled and applied to a hydroxyapatite column (1 x 6 cm) equilibrated with 5 mM potassium phosphate buffer (pH 7.5) containing 0.2 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 0.2 mM PMSF washed with 25 mM potassium phosphate buffer (pH 7.5). The elution was carried out with 40 mL of a linear gradient from 25 mM to 250 mM potassium phosphate buffer, active fractions were collected, concentrated and desalted with 25 mM Tris-Cl (pH 7.5), 0.2 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 0.2 mM PMSF, 10% glycerol in an Amicon Centricon-10. Sometimes the concentrated sample was frozen with liquid nitrogen and stored at -70°C. All purification procedures were carried out at 4°C or on ice.

**Enzyme Assay**

Protein kinase was assayed by measuring the incorporation of $^{32}$P from [γ-$^{32}$P]ATP into histone H1 type III-S. The standard assay mixture (50 μL) contained 25 mM Tris-Cl (pH 7.5), 1 mg/mL histone H1, 10 mM MgCl$_2$, 1 mM EGTA, 100 μM free CaCl$_2$, 100 μM ATP (400-1200 cpm/μmol), and 10 μL enzyme solution. The free Ca$^{2+}$ concentration for this assay buffer and for the other Ca$^{2+}$/EGTA buffers used was calculated by a computer program based on Fabiato (9). For experiments testing Ca$^{2+}$ activation of the protein kinase, all glassware, pipet tips, and microcentrifuge tubes were washed with warm water, soaked in 10 mM EDTA for 24 h, then washed thoroughly with Milli-Q water. The concentrations of calmodulin, PS, DO, W$_s$, W$_c$, and sphingosine were present as indicated in the table and figure legends. The reactions were started by adding 1.25 μL [γ-$^{32}$P]ATP. Assay mixtures were incubated at 25°C for 15 min. The reactions were terminated by spotting 30 μL of reaction mixture onto P81 (Whatman) paper discs, then dropping the discs into 75 mM H$_3$PO$_4$ (10 mL/disc). After washing three times in H$_3$PO$_4$ (5 min each wash), the discs were washed with methanol for 1 min, followed by ethyl ether for 30 s, then air dried. Radioactivity was determined by liquid scintillation counting.

**Detection of the Protein Kinase Activity in Gels after SDS-PAGE**

The protein kinase activity was detected in SDS-PAGE according to the method described by Geahlen et al. (10), with some modifications. The enzyme samples were added to SDS sample buffer without boiling and electrophoresed on SDS-PAGE as described by Laemmli (17) except that 1 mg/mL of histone H1 was added to the running gel before polymerization. SDS was removed by washing the gel with 25 mM Tris-Cl (pH 7.5) buffer for 30 min three times at room temperature immediately after electrophoresis. The enzyme was allowed to reature in 25 mM Tris-Cl (pH 7.5), 0.5 mM DTT at 4°C for 20 to 24 h. The gel was then incubated at room temperature for 10 to 15 h with gentle shaking in 20 mL of standard enzyme assay mixture except that the ATP concentration was 2.5 mM (200 μCi). The reaction was stopped by addition of stopping solution containing 5% TCA, and 1% NaPPi after removing the reaction mixture. The gel was washed extensively with the same solution, then stained with Coomassie blue and destained. For autoradiography, the dried gel was exposed to Kodak XAR-5 film at -70°C.

**SDS-PAGE**

Electrophoresis was carried out according to the method of Laemmli (17). Phosphorylation of endogenous proteins was performed as in the standard assay. The reactions were terminated by adding SDS sample buffer and the products were electrophoresed on SDS-PAGE. The phosphorylated proteins were detected by autoradiography of the dried gels. The possibility that the kinase would show a mobility shift in the presence of Ca$^{2+}$ or EGTA in SDS-PAGE was tested using the methods of Harmon et al. (12) and Bogre et al. (5).

**IEF Gel Electrophoresis**

A concentrated enzyme sample that had been purified through the hydroxylapatite step was subjected to analytical flat gel IEF over a pH range from 3 to 10 (IsoGel FMC). The samples were loaded about 2 cm from the negative pole and focused for 90 min at 500 V, 5°C. When the focusing was complete, the gel slab was cut into several lanes. One lane with pl standards was fixed, stained with Coomassie blue, and destained. The protein from other lanes were transferred to nitrocellulose membrane by contact diffusion for about 20 min in a transfer buffer that included 20 mM Tris, 500 mM NaCl (pH 7.5).

**Localization of the Kinase after Transfer to Nitrocellulose Membrane**

The blots of kinase samples that had been subjected to IEF and transferred to nitrocellulose were washed with 140 mM NaCl, 10 mM Tris (pH 7.5) 3 x 10 min then incubated in the assay mixture used for the detection of the protein kinase activity in gels after SDS-PAGE, either in the presence of 100 μM Ca$^{2+}$ or in the presence of 1 mM EGTA. The reaction was stopped after 2 h, with stopping solution (5% TCA, 1% NaPPi), then the blots were washed extensively and air dried. Labeled protein bands were detected by autoradiography.

**Immunodetection of Endogenous Calmodulin after Transfer to Nitrocellulose Membrane**

A protein blot from an IEF gel was fixed in 0.2% glutaraldehyde in PBS (pH 7.3), washed 3 x 5 min in PBS and blocked in 5% dry milk containing 0.05% Tween 20 and 0.5%
mg/mL Na Azide. The blot was incubated with rabbit antispinach calmodulin IgG (obtained from Dr. Linda Van Eldik) then incubated with secondary antibody (goat anti-rabbit IgG; alkaline phosphatase conjugate, Sigma) and the color reaction was developed with Sigma 104 phosphatase substrate according to instructions given by the manufacturer.

**Immunoblots of the Protein Kinase**

A kinase preparation purified through the hydroxylapatite step and CDPK (a generous gift from Dr. A. Harmon) were electrophoresed on SDS-PAGE and electroblotted onto nitrocellulose membranes. The protein blots were immunostained with monoclonal antibodies directed against the soybean CDPK (gift from A. Harmon) according to the methods used by Putnam-Evans et al. (25). The secondary antibody was phosphatase-labeled goat anti-mouse IgG(γ) (Kirkegaard & Perry Laboratories, Inc.).

**Protein Determination**

Protein concentrations were determined with a Bio-Rad protein assay kit using BSA as standard.

**RESULTS**

The Ca²⁺-dependent protein kinase was partially purified from *Dunaliella salina* by a combination of ammonium sulfate fractionation, Ca²⁺-dependent hydrophobic interaction chromatography on Phenyl-Sepharose and hydroxylapatite column chromatography. A summary of the purification is presented in Table I. In the fraction precipitated by ammonium sulfate, both Ca²⁺-dependent and -independent protein kinase activities were detectable. Chromatography of this crude extract on a Phenyl-Sepharose column revealed a protein kinase which required Ca²⁺ both for binding to the column and for its activity. A typical elution profile is shown in Figure 1A. In the presence of EGTA, the major protein peak eluted showed almost no activity, indicating that there

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**Table I. Summary of the Purification**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total Protein</th>
<th>Activity</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>mmol/min</td>
<td>-fold</td>
</tr>
<tr>
<td>50% (NH₄)₂SO₄ precipitate</td>
<td>195</td>
<td>11.3</td>
<td>0.06</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>1.75</td>
<td>5.9</td>
<td>3.38</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>0.39</td>
<td>3.1</td>
<td>7.84</td>
</tr>
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* Difference between total activities assayed in the presence of Ca²⁺ (100 μM) and in the presence of EGTA (1 mM). The enzyme assay was carried out in the standard conditions. (See "Materials and Methods" for details).

Figure 1. Elution profiles of protein kinase from A, Phenyl-Sepharose column after Ca²⁺-dependent, hydrophobic interactive chromatography; B, Hydroxylapatite column chromatography. Protein kinase activity was assayed in duplicate under standard conditions. Each point represents the mean of the duplicate assays. Protein kinase activity in the presence of 100 μM Ca²⁺ (■); protein concentration (○); salt concentration (— — —).
was very little Ca\textsuperscript{2+}-independent protein kinase activity present under the assay conditions. Further purification of the sample on a hydroxylapatite column also resulted in a single protein kinase activity peak (Fig. 1B), less than 3% of which was Ca\textsuperscript{2+}-independent. The hydroxylapatite-purified sample was analyzed on SDS-PAGE and IEF, and was shown not to be homogeneous (Fig. 2). When the kinase activity was localized on the gel by in-situ gel localization methods, its position corresponded to a $M_r$ 40,000 protein band on the SDS-PAGE gel and a doublet with pl 6.7 (major) and 7.1 (minor) on the IEF gel. The minor band could barely be seen with Coomassie blue staining, but could be easily detected by autoradiography. The amount of protein in the doublet bands was about 7% of total protein-staining material on the gel, based on density scanning of the stained bands on the dried IEF gel. In contrast, about 20% of the total protein-staining material on the gel after SDS-PAGE was at the position of the kinase ($M_r$ 40,000). However, two-dimensional gel analysis of the sample revealed four different protein-staining spots at $M_r$ 40,000, so 7% is probably a more reasonable estimate of the sample purity. The apparent $M_r$ of the kinase was not affected by the presence or absence of Ca\textsuperscript{2+} in the electrophoresis buffer (data not shown).

Some properties of the protein kinase were examined using histone H1 type III-S as substrate. Casein was a poor substrate when it was substituted for histone. The kinase requires Mg\textsuperscript{2+} for its activity in addition to Ca\textsuperscript{2+}. Maximal activity is obtained at 5 mM Mg\textsubscript{2+} with higher concentrations being inhibitory. Tests of the pH dependence of the kinase (Fig. 3) showed that the optimum pH was 7.5 under the assay conditions used. The enzyme was very sensitive to Ca\textsuperscript{2+} activation (Fig. 4). Histone H1 phosphorylation was activated above $10^{-7}$ M, with half-maximal activation at $3 \times 10^{-7}$ M, and maximal activation between $10^{-6}$ and $10^{-4}$ M.

To investigate the effects of calmodulin and phospholipids on enzyme activity, several experiments were carried out. The lipids, PS, DO, or both together had no obvious effect on enzyme activity and did not change the Ca\textsuperscript{2+} sensitivity of the enzyme (Fig. 4). The slight alterations of the enzyme activity shown in Figure 4 were not significant. Sphingosine, a PKC inhibitor in animal cells (11), had little effect on enzyme activity: the inhibition caused at 800 $\mu$M was less than 30% (Table II). On the other hand, the enzyme activity was inhibited by the calmodulin antagonists, W\textsubscript{5} and W\textsubscript{7}, in a dose-dependent manner over a range between $10^{-3}$ and $10^{-1}$ M (data not shown). The inhibition effect of W\textsubscript{7} was stronger than that of W\textsubscript{5} (Table II).

The exogenous addition of spinach calmodulin up to 1 $\mu$M did not significantly increase protein kinase activity (Table II), although occasionally a slight stimulation was observed. The effect of calmodulin seems to be nonspecific. However,
immunoassay showed some calmodulin was present in the preparation (Fig. 5B), so it became important to separate calmodulin from the kinase to determine whether or not this was required for the calcium-stimulated activity. Efforts to do so by DEAE chromatography were not successful. As an alternative approach, calmodulin and the kinase were separated by IEF in the presence of EGTA. After blotting the separated proteins onto nitrocellulose, the kinase activity was localized on the blot, and the position of calmodulin was located by immunostain. The kinase activity appeared as a doublet at pH 6.7 and 7.1 (Fig. 5A); the calmodulin immunostain also appeared as a doublet at pH 3.9 and 4.1 (Fig. 5B). After this separation from calmodulin, both histone kinase activity (data not shown) and phosphorylation of endogenous protein showed stimulation by Ca\(^{2+}\) (Fig. 5A), indicating that this stimulation was calmodulin-independent.

When phosphorylation was carried out in the hydroxylapatite-purified preparation and then subjected to SDS-PAGE analysis, the presence of phosphorylated bands at positions in addition to that at Mr 40,000 could be detected (Fig. 5A). This indicated that the kinase could phosphorylate some of the endogenous protein substrates that copurify with it.

Further indication of the phospholipid and calmodulin independence of the kinase was the fact that at every step in the purification it could carry out in-gel phosphorylation after SDS-PAGE and renaturation in the gel (Fig. 6). This could be demonstrated even in experiments in which the lower part of the gel (where separated phospholipids and calmodulin would be expected to migrate) was cut off immediately after the electrophoresis to eliminate the possibility of these agents diffusing to the kinase during the renaturation steps.

To investigate the relationship between CDPK described in higher plants (12) and the Dunaliella kinase, the enzyme sample and soybean CDPK were immunoassayed by Western blot. The protein blots were immunostained with monoclonal antibodies (mixture of 14G5, 3B9, 3E8, and 12G8, all generously donated by Dr. A. Harman) directed against soybean CDPK. The CDPK used as a positive control gave a very strong reaction while no cross-reaction was observed in the kinase sample in the region of Mr 40,000 (Fig. 7). Because we estimate the Dunaliella kinase sample was more than 5% pure, there should have been more 40 kD kinase from Dunaliella on the blot than there was CDPK protein.

**DISCUSSION**

The protein kinase described here is highly Ca\(^{2+}\)-sensitive, with activation at micromolar free Ca\(^{2+}\) concentrations. As such it has the potential to serve as a stimulus-response coupler in Ca\(^{2+}\)-regulated cell processes in Dunaliella. Specifically, it could be an important participant in the well-described responses of *D. salina* to osmotic stress, because these responses are known to involve the increased turnover of membrane inositol phospholipids (6, 7), which, in turn, is often coupled to increases in intracellular Ca\(^{2+}\).

This Ca\(^{2+}\)-dependent protein kinase is phospholipid independent. This conclusion follows from several different observations. First of all, the addition of phospholipids had no effect on enzyme activity. Since different isoforms of PKC in animal cells have different cofactor requirements at different free Ca\(^{2+}\) concentrations (16), the effect of PS and DO was examined at different Ca\(^{2+}\) concentrations (Fig. 4). No apparent effect was observed. Furthermore, when the PKC inhibitor sphingosine was added at various concentrations up to 300 μM, the activity only decreased slightly, a result similar to that reported for phospholipid-independent protein kinases examined in animal cells (11). Sphingosine is thought to inhibit PKC by interfering with phospholipid binding to the kinase (11, 22). The failure of sphingosine to inhibit the protein kinase implies, in our case, that the enzyme activity does not require phospholipids. Detection of kinase activity in the enzyme after SDS-PAGE strongly supports this conclusion.

**Table II.** Effects of Various Agents on Ca\(^{2+}\)-Dependent Kinase Activity

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration (μM)</th>
<th>Relative Activity % of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphingosine</td>
<td>800</td>
<td>74</td>
</tr>
<tr>
<td>W7</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>Ws</td>
<td>100</td>
<td>40</td>
</tr>
<tr>
<td>K-252a</td>
<td>1</td>
<td>90</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>0.05</td>
<td>50</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>1</td>
<td>105</td>
</tr>
</tbody>
</table>

*Values are the means of duplicate determinations. The enzyme activity was determined in the presence of 100 μM Ca\(^{2+}\) under standard assay conditions.*
The protein kinase does not seem to be regulated by calmodulin either, since exogenous calmodulin did not increase the enzyme activity. Additionally, the enzyme showed activity after SDS-PAGE in gels which almost definitely did not contain calmodulin, and after IEF in blots where the kinase could be demonstrated to be free of calmodulin. The protein kinase was inhibited by Ws, W2 in a similar manner to that of calmodulin-regulated enzymes (4, 14, 15). This result does not counter the conclusion that the enzyme is calmodulin-independent, since many other calmodulin-independent protein kinases that are regulated by Ca2+ are inhibited by these drugs (1, 5, 12).

Both calmodulin and the Ca2+-dependent kinase appeared to be heterogeneous after analysis by isoelectric focusing (Fig. 5). Others have noted and discussed reasons for the heterogeneity of calmodulin (23); the heterogeneity of the kinase could be due to the same cause: posttranslational modifications. However, further analyses of the kinase will be required to test this possibility and reveal the nature of these modifications, if they occur. Alternatively, Dunaliella may have two different 40 kD Ca2+-dependent kinases, both of which are calmodulin- and phospholipid-independent.

The apparent mol wt of the protein band which showed the protein kinase activity was 40,000 as judged by SDS-PAGE. A protein band of the same $M_r$, showed kinase activity through every purification step, indicating that it is unlikely the kinase was proteolytically degraded during purification. The kinase could be a monomer with a native mol wt of 40,000, or the 40 kD protein could be only the catalytic subunit of a multimeric enzyme. If this protein kinase is monomeric, it is smaller than the CDPKs from soybean and alfalfa, which are 46 to 51 kD and 50 to 65 kD, respectively.

The Dunaliella CDPK differs from the one in soybean, not only in molecular weight, but also in its antigenic properties. The failure of the Dunaliella kinase to cross-react with any of the four monoclonal antibodies that recognize the soybean CDPK suggests that these two calcium-dependent kinases differ significantly in their primary structure.
Figure 7. Immunoblot analysis of the protein kinase. The enzyme sample from hydroxylapatite column (6 μg, lane 2) and soybean CDPK (0.25 μg, lane 1) were subjected to 10% SDS-PAGE and transferred onto nitrocellulose membranes the blots were immunostained with monoclonal antibodies directed to soybean CDPK.

We were unable to demonstrate the mobility shift of the protein kinase in the presence of Ca\(^{2+}\) or EGTA in SDS-PAGE under conditions in which spinach calmodulin, which was used as a positive control, did show a mobility change (data not shown). No mobility shifts were seen among the several protein bands that co-purified with the 40 kD kinase band in the preparation. Since the mobility shift is a major property of CDPKs and some Ca\(^{2+}\)-binding proteins, the question is raised whether the 40 kD protein itself is a Ca\(^{2+}\)-binding protein. The behavior of the protein kinase on a Phenyl-Sepharose column is very similar to that of calmodulin. That is, it binds to the column in a Ca\(^{2+}\)-dependent manner, and washing the column extensively with both low (no NaCl) and high salt (1 M NaCl) concentrations in the presence of Ca\(^{2+}\) does not release the enzyme. However, it is eluted with EGTA rapidly. This result is highly reproducible, but alone it is not an adequate criterion for determining whether the 40 kD protein is a Ca\(^{2+}\)-binding protein. The calcemedins are examples of Ca\(^{2+}\)-binding proteins that do not show Ca\(^{2+}\) dependent M\(_k\) shifts on SDS-PAGE (19). The discrepancy between the enzyme and CDPKs in the aspect of the gel mobility shift may be intrinsic or experimental, and this needs to be examined further.

Because the phosphorylation shown in Figure 5 occurred with no exogenous substrate added, a possible interpretation of these results is that the 40 kD kinase from \textit{Dunaliella} is capable of autophosphorylation. Many protein kinases are capable of autophosphorylation, and this property may be an important way to regulate the enzyme activity (18). Very limited information is known about this aspect of kinases, especially in plant cells. Recently, it was found that autophosphorylated CDPKs had increased activity (5). Whether our protein kinase has a similar property remains to be tested. Details on the occurrence and function of autophosphorylation of plant protein kinases \textit{in vivo} are not known.

Even after hydroxylapatite column chromatography, the kinase preparation is still far from homogeneous. However, it should be feasible to electroelute enough of the IEF-purified kinase to both raise antibodies to the enzyme and obtain partial sequence information on it (2), and this work is in progress. The antibodies and sequence data will allow us to more rigorously evaluate the relationship of the \textit{Dunaliella} kinase to the CDPKs that have been purified from higher plants and from animals.

\section*{ACKNOWLEDGMENTS}

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\section*{LITERATURE CITED}

1. Battey NH, Venis MA (1988) Calcium-dependent protein kinase from apple fruit membranes is calmodulin-independent but has calmodulin-like properties. Planta 176: 91–97
polyacrylamide gels. Anal Biochem 153: 151–158
11. Hammun YA, Loomis CR, Merrill AH Jr, Bell RM (1986) Sphingo-
sine inhibition of protein kinase C activity and of phosphor-
dibutylate binding in vitro and in human platelets. J Biol
Chem 261: 12604–12609
dependent but calmodulin-independent protein kinase from
soybean. Plant Physiol 83: 830–837
tionship of calmodulin antagonists: naphthalenesulfonamide
derivatives. Mol Pharmacol 20: 571–578
15. Hidaka H, Yamaki T, Naka V, Tanaka T, Hayashi H, Kob-
abayashi K (1980) Calcium-regulated modulator protein inter-
acting agents inhibit smooth muscle calcium-stimulated pro-
17. Laemmli UK (1970) Cleavage of structural proteins during the
685
of Ca2+/calmodulin-dependent protein kinase II by Ca2+/cal-
modulin-independent autophosphorylation. J Biol Chem 263:
19232–19239
inum-dependent hydrophobic binding proteins: possible in-
dependent mediators of intracellular calcium distinct from
alterations in the chloroplast and microsomal membranes of
C and diverse cell functions by sphingosine—a pharmacologi-
cally interesting compound linking sphingolipids and signal
transduction. Biochim Biophys Acta 1010: 131–139
23. Murtaugh TJ, Rowe PM, Vincent PL, Wright LS, Siegel FL
Enzymol 102: 158–170
ization of a second calmodulin-activated Ca2+-dependent pro-
tein kinase from wheat germ. Biochim Biophys Acta 785: 68–
74
25. Putnam-Evans C, Harmon AC, Palevitz BA, Fechheimer M,
Cormier MJ (1989) Calcium-dependent protein kinase is lo-
calized with F-actin in plant cells. Cell Motil Cytoskeleton 12:
12–22
plants; regulatory effects and potential involvement in stimu-
kinase in Mougeotia by using synthetic peptide substrates.
Plant Physiol 91: 1613–1619
stimulated gravitropism: role of calcium. CRC Crit Rev Plant
Sci 5: 205–236
regulation by calcium and calmodulin in membrane fractions
from zucchini hypocotyls. Planta 158: 560–568
30. Schafer A, Bygrave F, Matzenaver S, Marme D (1985) Identifi-
cation of a calcium and phospholipid-dependent protein kinase