A Calcium-Dependent but Calmodulin-Independent Protein Kinase from Soybean

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ABSTRACT

A calcium-dependent protein kinase activity from suspension-cultured soybean cells (Glycine max L. Wayne) was shown to be dependent on calcium but not calmodulin. The concentrations of free calcium required for half-maximal histone H1 phosphorylation and autophosphorylation were similar (≈2 micromolar). The protein kinase activity was stimulated 100-fold by ≥10 micromolar free calcium. When exogenous soybean or bovine brain calmodulin was added in high concentration (1 micromolar) to the purified kinase, calcium-dependent and -independent activities were weakly stimulated (≈2-fold). Bovine serum albumin had a similar effect on both activities. The kinase was separated from a small amount of contaminating calmodulin by sodium dodecyl sulfate polyacrylamide gel electrophoresis. After renaturation the protein kinase autophosphorylated and phosphorylated histone H1 in a calcium-dependent manner. Following electrophoresis onto nitrocellulose, the kinase bound "Ca2+" in the presence of KCl and MgCl2, which indicates that the kinase itself is a high-affinity calcium-binding protein. Also, the mobility of one of two kinase bands in SDS gels was dependent on the presence of calcium. Autophosphorylation of the calmodulin-free kinase was inhibited by the calmodulin-binding compound N-(6-aminohexyl)-S-chloro-1-naphthalene sulfonamide (W-7), showing that the inhibition of activity by W-7 is independent of calmodulin. These results show that soybean calcium-dependent protein kinase represents a new class of protein kinase which requires calcium but not calmodulin for activity.

One of the ways in which Ca2+ may play a second messenger role in plants is to activate protein kinases in response to stimuli such as growth regulators, light, or stress (5). The receptor and transducer of the calcium signal could be the Ca2+-binding protein calmodulin or the protein kinase itself. There have been several reports of calcium/calmodulin-dependent protein kinases (1, 20, 21, 23, 25, 31) and calcium/phospholipid-dependent protein kinases (17, 26) in plants. Most of the evidence for calmodulin dependence has been indirect, i.e. based on inhibition studies with calmodulin antagonists and on activation studies with exogenous calmodulin performed with impure protein kinase preparations. None of these protein kinases has been purified to homogeneity and their interaction with calmodulin demonstrated.

Previously we partially purified a calcium-dependent protein kinase from suspension-cultured soybean cells (22). This CDPK2 was completely inhibited by 1 mM W-7, a calmodulin-binding compound, and was activated a slight amount (30%) by micromolar concentrations of calmodulin (22). Because the CDPK preparation was contaminated with calmodulin and because such high concentrations of W-7 and calmodulin were required for an effect, we were unable to conclude whether calmodulin was the regulatory of CDPK. To demonstrate whether CDPK is dependent on calmodulin for calcium sensitivity and activity, we have purified the enzyme 1000-fold. We show here that CDPK directly binds calcium and is not dependent on calmodulin for activity.

MATERIALS AND METHODS

Chemicals. W-7 and W-5 were synthesized according to Hidaka et al. (9) and Hart et al. (8). Magnesium chloride (Analar grade) from BDH Chemical Co. and 100 mM stock calcium solution from Orion were used in calcium buffers. 45CaCl2 (335 Ci/mol) was obtained from ICN and [γ-32P]ATP was from Amersham.

Plant Material. Soybean (Glycine max L. Wayne) cell suspension cultures were originally obtained from Dr. Joe L. Key, Department of Botany, University of Georgia. The cells were subcultured weekly and grown in the dark at 30°C and 175 rpm in Murashige and Skoog medium (16) supplemented with 20 g/L sucrose, 1 mg/L naphthaleneacetic acid, and 0.5 mg/L kinetin.

Proteins. Calcium-dependent protein kinase was purified 1000-fold (0.9 μmol 32P transferred per min per mg of enzyme) from suspension-cultured soybean cells by chromatography on DEAE-Cellulose, TAPP-Sepharose, Sephadex G-100, and Cibacron Blue-Sepharose. Procedures for chromatography on the first three resins have been described (22). The purification and characterization of CDPK will be described elsewhere.

Apoaenaurin was a gift of Miss Judy C. Peek of our laboratory. Sheep anticalmodulin IgG was a gift of Dr. Anthony Means, Baylor College of Medicine. Bovine brain calmodulin was prepared by the method of Charbonneau et al. (4). Soybean cell calmodulin co-purified with CDPK through the first three chromatography steps. Calmodulin was separated from CDPK by chromatography on Cibacron Blue-Sepharose. Calmodulin did not bind to the resin and was recovered in the flow-through fractions. This calmodulin was ≥95% pure as shown by SDS-PAGE and was able to fully activate the calmodulin-dependent enzymes pea NAD kinase and human erythrocyte Ca2+-ATPase (data not shown).

Pea seedling NAD kinase was purified through the carboxymethyl cellulose step according to Harmon et al. (7). Histone H1 (Sigma Chemical Co., type III-S) was exhaustively dialyzed against deionized H2O to remove contaminating calcium.

Protein Determination. The concentration of a stock solution of soybean calmodulin in deionized H2O was determined from amino acid analysis of a 24 h hydrolysate. The amino acid composition of spinach calmodulin (14) was used for the calcu-
lution of concentration. The concentrations of all other protein solutions were determined with a Bio-Rad protein assay kit which is based on the method of Bradford (2).

Enzyme Assays. The free calcium concentration in the protein kinase and autophosphorylation assays was controlled by a Ca$^{2+}$/EGTA buffer (final concentrations: 0.2 mM EGTA, 10 mM MgCl$_2$, 50 mM Heps [pH 7.2], and various amounts of calcium stock solution). The resulting free calcium concentrations were calculated by a computer program based on the method of Perrin and Sayce (19). The stability constants reported by Sillén and Martell (29) for metal-chelator and metal-ATP complexes were used in the calculations.

Protein kinase activity was determined by measuring incorporation of labeled phosphate from [$\gamma$-$32$P]ATP into histone H1. The standard assay mixture contained, in a volume of 0.15 ml, 1 mg/ml histone H1, 470 ng/ml CDPK, and Ca$^{2+}$/EGTA buffer and the reaction was initiated by the addition of 50 $\mu$M [$\gamma$-$32$P]ATP (240 cpm/pmol). For some experiments CDPK was incubated overnight at 4°C with anticalmodulin IgG or nonimmune IgG in Ca$^{2+}$/EGTA buffer (no added calcium) prior to assay. Assay mixtures were incubated at 30°C for 6 min and the reactions were terminated by the addition of 0.5 ml of cold 20% TCA containing 0.2% sodium pyrophosphate. The precipitates were collected on Whatman GFA glass filters and washed with 20% TCA followed by ethanol/ether (1:1). The filters were added to scintillation vials containing 2 ml Scintiverse II (Fisher Scientific) and counted in a liquid scintillation counter.

For autophosphorylation assays, CDPK was incubated for 1 min at 30°C in a total volume of 25 $\mu$l. The reaction mixture contained Ca$^{2+}$/EGTA buffer, 1 $\mu$M [$\gamma$-$32$P]ATP (7500 cpm/pmol), 0.1 mg/ml BSA, and various concentrations of calcium. Reactions were terminated and the $32$P-incorporation was determined as described for the protein kinase assay. For some experiments CDPK was autophosphorylated prior to electrophoresis. CDPK (5 $\mu$g) was incubated 5 min at 25°C in a total volume of 25 $\mu$l which contained EGTA buffer, 30 $\mu$M [$\gamma$-$32$P]ATP (2000 cpm/pmol). The reaction was terminated by addition of electrophoresis sample buffer (2% SDS, 1 mM EGTA, 2% glycerol, 5% 2-mercaptoethanol, 0.001% bromphenol blue, final concentrations). The samples were boiled for 2 min and electrophoresed as described below. The dried gels were autoradiographed with Kodak XAR-5 film and a DuPont Cronex Lightning Plus intensifying screen at -80°C.

The calmodulin-dependent enzyme NAD kinase was used to assay calmodulin. NAD kinase activity was measured as described by Harmon et al. (7) except that the change in A$_{450}$ was measured with a Varian DMS 100 Spectrophotometer equipped with a programmable cell changer. Soybean calmodulin was used as a standard. Samples of CDPK, histone H1, and BSA were boiled in EGTA-containing buffer prior to assay for calmodulin.

SDS-PAGE. Protein samples were electrophoresed by the method of Laemmli (13) in either 0.15 × 8.3 × 6 cm, 10% or 12% gels or in 0.15 × 14 × 12 cm, 10 to 15% gradient gels. $\lambda$-o was calculated from a plot of $R_g$ versus Log M, for the following marker proteins (Pharmacia low mol wt calibration kit): phosphorylase b, 94 kD; BSA, 83 kD; ovalbumin, 43 kD; carbonic anhydrase, 30 kD; soybean trypsin inhibitor, 20.1 K D; $\alpha$-lactalbumin, 14.4 kD. Proteins were detected by staining with Coomassie brilliant blue R.

Calcium-Binding Activity. Calcium-binding proteins were detected by $^{45}$Ca autoradiography of proteins which had been electrophoresed onto nitrocellulose following SDS-PAGE as described by Maruyama et al. (15). Following electrophoretting the nitrocellulose was washed three times for 20 min each in 60 mM KCl, 5 mM MgCl$_2$, 10 mM imidazole (pH 6.8). It was then incubated for 10 min in the same buffer to which 1 mM CaCl$_2$ carrier-free $^{45}$CaCl$_2$ had been added, washed in deionized H$_2$O for 5 min, and air-dried. Proteins bound to nitrocellulose were detected by staining with amido black (15).

Detection of Protein Kinase Autophosphorylation and Activity in SDS-Polyacrylamide Gels. A modification of the method of Geahlen et al. (6) was used to detect protein kinase autophosphorylation in SDS-polyacrylamide gels. The samples were heated to 80 to 90°C for 2 min in electrophoresis sample buffer (13) that contained SDS and 2-mercaptoethanol. The samples were electrophoresed as described (13) except that 0.1 mg/ml BSA was included in the separating gel before polymerization and the stacking gel was photopolymerized with 0.5 mg/L riboflavin as catalyst. After removal of the SDS with five changes of 50 mM Heps (pH 7.2), the gel was cut into vertical strips, each containing CDPK and M, markers. The strips were incubated in 50 ml Ca$^{2+}$/EGTA buffer, 30 $\mu$M [$\gamma$-$32$P]ATP (100-200 cpm/pmol) and either no added calcium, 13 $\mu$M-free calcium, or 13 $\mu$M-free calcium and 500 $\mu$M W-7. The slices were washed and fixed as described by Geahlen et al. (6).

For measurement of histone-phosphorylating activity, 5 $\mu$g CDPK was electrophoresed in each of two adjacent lanes in a 12.5% polyacrylamide gel as described for autophosphorylation above. The lanes were cut vertically with a cutter that left a scalloped edge on the gel. The protein in one of the gel strips was stained with Coomassie blue for 30 min, destained for 4 h, then incubated in 50 mM Heps (pH 7.2), for 30 min. Meanwhile, the protein in the other gel strip was renatured as described above for autophosphorylation. The paired strips were aligned by matching the scalloped edges and the portion of the gel corresponding to the 46 to 51 kD stained proteins was excised. A gel slice was prepared in this manner for each assay condition. The gel slices were added to 0.4 ml of protein kinase assay buffer in a 1:1.5 Eppendorf microfuge tube and the gel was crushed with a pestle (Kontes Scientific Glassware/Instruments). The tubes were inverted end-over-end for 3 h on a tube rotator (Scientific Equipment Products). An aliquot of the supernatant was removed, added to electrophoresis sample buffer, and subjected to SDS-PAGE. Phosphorylated histone H1 was detected by autoradiography.

RESULTS

Purity of CDPK. A CDPK which catalyzes the phosphorylation of the artificial substrate Histone H1 was purified 1000-fold from suspension-cultured soybean cells as described in “Materials and Methods.” CDPK was analyzed by SDS-PAGE to assess its purity (Fig. 1). The heavily protein-stained band centered at M, 48 kD in lane 2 was shown to be a doublet of M, = 46 and 50 kD when a lower amount of protein was loaded (lane 1) and in 10 to 15% gradient gels (Fig. 3A). We show below that these bands possess calcium-dependent protein kinase activity. These bands represent 80% of the protein in the preparation as determined by scanning densitometry of the stained gel (data not shown). Other prominently-stained bands observed in CDPK (Fig. 1) lane 2 had M, = 72-74, 41, and 32 kD. A faint band corresponding to soybean calmodulin (lane 3, M, = 17 kD) was observed in the overloaded CDPK sample in lane 2.

Caldulin in the CDPK preparation and in the histone H1 and BSA used in protein kinase assays was measured with a sensitive enzymic assay based on the activation of NAD kinase. Boiled aliquots of CDPK contained 18 ng calmodulin per $\mu$g of protein. No calmodulin was detected in histone H1 or BSA. In the standard protein kinase assay 470 ng/ml of CDPK was used, therefore the calmodulin concentration was 0.5 nm. Assuming a molecular mass of 48 kD and 80% purity for CDPK, the concentration of the enzyme in the assay mixture was 8 nm.

Calcium-Dependence of Protein Kinase Activity and of Autophosphorylation. The phosphorylation of histone H1 by CDPK was stimulated 100-fold by the addition of ≥10 $\mu$M-free calcium (Fig. 2; Table I). No exogenous calmodulin was required for this effect of calcium. Phosphorylation was half maximal at 2.1 $\mu$M-free calcium, and...
Autophosphorylation was also stimulated by the addition of free calcium (Fig. 2B and Fig. 3). The \( V_{\text{max}} \) was 10-fold higher with calcium than without. The relative activity in the absence of calcium was higher for autophosphorylation than for histone phosphorylation. The concentration of calcium (\( \approx 2 \mu M \)) required for stimulation of autophosphorylation was similar to that required for histone H1 phosphorylation (Fig. 2A). The autoradiogram in Figure 3 shows that calcium promoted the incorporation of labeled phosphate into at least two protein bands in the 46 to 51 kD range. Other \( ^{32} \text{P} \)-labeled bands at 41 and 32 kD were visible when the autoradiogram was overexposed (data not shown).

The \( ^{32} \text{P} \) label associated with the protein bands of 46 to 51 kD was not diminished when, prior to electrophoresis, the autophosphorylated CDPK was successively precipitated with TCA, washed with acetone, and then treated with hydroxylamine (data not shown). These treatments destroy phosphohistidine and phosphosine, remove phospholipids, and destroy acylphosphate, respectively. This observation indicates that the phosphorylated residue(s) is serine, threonine, or tyrosine.

**Effect of Calmodulin on Protein Kinase Activity.** Since the amount of calmodulin present in the protein kinase assay was substoichiometric, based on the calculations above, the effect of exogenous calmodulin on CDPK activity was tested. When soybean calmodulin was added in large excess (1 \( \mu M \)) over the concentration of CDPK in the presence of calcium, a slight

\[ \text{FIG. 1. SDS-PAGE of CDPK. Lyophilized CDPK (6} \mu g \text{ in lane 1, 36} \mu g \text{ in lane 2) or soybean calmodulin (10} \mu g \text{ in lane 3) were electrophoresed in a 12% polyacrylamide gel in the presence of SDS. Protein was stained with Coomassie blue. The positions and} M_s \text{ in kD of the protein markers are indicated.} \]

\[ \text{FIG. 2. Effect of calcium on histone H1 phosphorylation and autophosphorylation.} \text{ A, The phosphorylation of histone H1 by 470 ng/ml CDPK was measured in the presence of various concentrations of free calcium. The point plotted on the ordinate is the activity with no added calcium. Each point is the average of duplicate assays and the bars indicate the standard deviation.} \text{ B, The autophosphorylation of CDPK (0.2 mg/ml) was measured in the presence of various concentrations of calcium. The point plotted on the ordinate is the fraction of maximal activity measured with no added calcium. Each point is the average of two determinations in duplicate and the bar indicates the standard deviation.} \]
experiments. The effect of bovine brain calmodulin on activity in the presence of calcium was similar to the effect of soybean calmodulin (Table 1).

The effect of calmodulin on CDPK activity appears to be nonspecific, since such high concentrations were required for activation and since both basal and Ca\(^{2+}\)-stimulated activities were affected. Furthermore, when 1.5 \(\mu\)M BSA was added to the protein kinase assay in the second experiment in Table I it also stimulated protein kinase activity in the presence and absence of calcium.

**Calcium-Independence of CDPK Autophosphorylation and Activity.** To directly demonstrate that CDPK autophosphorylation and activity were independent of calmodulin, CDPK was separated from calmodulin by electrophoresis in SDS gels, renatured, and either autophosphorylated in the gel or excised from the gel and incubated with the standard protein kinase assay mixture. Figure 4A shows the results of autophosphorylation of CDPK following SDS-PAGE. The proteins ranging in \(M_r\) from 46 to 51 kD were not resolved in this 10% polyacrylamide gel and migrated as a single band of \(M_r = 49\) kD. Labeled phosphate was incorporated into these proteins in a calcium-independent manner. To show that the labeling with \(^{32}\)P was covalent and not nonspecific, the bands were excised from the dried gel, incubated in electrophoresis sample buffer, and electrophoresed on a second SDS gel. The bands were again detected by autoradiography (data not shown).

To further examine which of the protein bands had autophosphorylated, CDPK was electrophoresed in a 10 to 15% SDS-gradient gel and then autophosphorylated in the gel (Fig. 4B). Comparison of the two bands that were autophosphorylated after SDS-PAGE (Fig. 4B) to the bands that were autophosphorylated when CDPK was incubated with [\(\gamma\)\(^{32}\)P]ATP prior to electrophoresis (Fig. 3B) shows that the same two bands were labeled in both cases.

The 46 to 51 kD proteins renatured following SDS-PAGE were capable of phosphorylating histone H1 in addition to autophosphorylating. When the renatured 46 to 51 kD proteins were excised in a single slice from an SDS gel, and incubated in the standard protein kinase assay mixture, they catalyzed the phosphorylation of histone H1 in a calcium-dependent manner (Fig. 5). Since no calmodulin was detected in the histone H1 or the BSA, the experiments in Figures 4 and 5 show that both the activity and the autophosphorylation of CDPK are calcium dependent but not calmodulin dependent.

The calmodulin independence of the histone-phosphorylating activity of CDPK was confirmed in experiments in which antibodies directed against calmodulin were included in the assay mixture. CDPK was preincubated with either nonimmune IgG or with an amount of anticalmodulin IgG that inhibited NAD-
kinase activity in the presence of up to 10 ng/ml soybean calmodulin. The activity of CDPK was slightly inhibited (10%) in the presence of calcium and inhibited 50% in the absence of calcium, whereas nonimmune IgG slightly stimulated activity. The addition of up to 1 µg/ml soybean calmodulin to the preincubation mixture in the presence of calcium did not restore activity (CP-Evans, AC Harmon, MJ Cormier, unpublished observations).

Ca\(^{2+}\)-Binding Activity of CDPK. CDPK and other proteins which had been electrophoresed onto nitrocellulose following SDS-PAGE were incubated with \(^{45}\)Ca\(^{2+}\) (Fig. 6). Protein bands of 46 kD and 50 kD in CDPK (lane 2) and the positive controls, soybean calmodulin (lane 3) and the Ca\(^{2+}\)-dependent photoprotein aequorin (Mr = 25 kD, lane 4), produced strong signals on the autoradiograms (lanes marked B). Bands of 61, 51, and 34 kD in CDPK (lane 2B) produced weak signals on the autoradiogram. The Mr marker proteins, which were used as negative controls, did not produce signals on the autoradiogram (lane 1), although in some experiments soybean trypsin inhibitor and α-lactalbumin produced weak signals (data not shown). BSA did not bind \(^{45}\)Ca\(^{2+}\) even when 15 µg of protein was loaded on the gel (data not shown). Calmodulin did not stain well with amido black (lane 3A). Calmodulin also does not stain well with Ponceau S or India ink (AC Harmon, MJ Cormier, unpublished observations).

Ca\(^{2+}\)-Dependent Mobility Change in SDS-PAGE. When CDPK was electrophoresed in SDS gels in the presence of 2 mM Ca\(^{2+}\) or 2 mM EGTA, different mobilities were observed for one of the Coomassie blue-stained bands (Fig. 7). The mobility of this band was greater in the presence of Ca\(^{2+}\) (Mr = 43 kD, Lane 2) than in the absence of Ca\(^{2+}\) (Mr = 46 kD, lane 3). The mobility of the 50 kD band was unaffected.

Calmodulin-Independence of the Inhibition of CDPK Auto- phosphorylation and Histone H1-Phosphorylating Activity by W-7 and W-5. The naphthalene sulfonamide W-7, which is a calmodulin-binding compound, and its less potent analog W-5 were both able to inhibit the phosphorylation of histone H1 by CDPK (Fig. 8). The concentrations of W-7 and W-5 required for 50% inhibition were 110 µM and ≈ 1 mm, respectively. W-7 also inhibited autophosphorylation of CDPK which had been separated from calmodulin by electrophoresis in an SDS-polyacrylamide gel (Fig. 4A, +Ca\(^{2+}\) +W-7). In the presence of calcium and 500 µM W-7 the incorporation of labeled phosphate was comparable to the labeling in the absence of calcium (Fig. 4A, -Ca\(^{2+}\)). This indicates that W-7 inhibits enzyme activity by interacting directly with the enzyme and not with calmodulin.

**DISCUSSION**

The protein bands on one-dimensional SDS-polyacrylamide gels that are associated with soybean calcium-dependent protein kinase have been identified. At least two bands in the Mr = 46 to 51 kD range were shown to autophosphorylate (Fig. 4) and to phosphorylate histone H1 (Fig. 5) in a calcium-dependent manner following SDS-PAGE. Several other bands in the CDPK preparation incorporated \(^{32}\)PO\(_4\) when CDPK was incubated with \([γ-^{32}\text{P}]\text{ATP}\) prior to SDS-PAGE (Fig. 3). These results indicate that catalytic activity is associated with the 46 to 51 kD proteins and that the other proteins are either contaminants or noncatalytic subunits of CDPK that can serve as substrates of the enzyme.

**Fig. 4.** Autophosphorylation of CDPK following SDS-PAGE. CDPK (5 µg) was electrophoresed in a 10% SDS-polyacrylamide gel, renatured in the gel, and autophosphorylated in the presence of 13 µM-free Ca\(^{2+}\) (+Ca\(^{2+}\)), in the absence of calcium (−Ca\(^{2+}\)), or in the presence of 13 µM Ca\(^{2+}\) and 0.5 µM W-7 (+Ca\(^{2+}\), +W-7). A, Autoradiographs of the dried gels. The positions and Mr of the marker proteins are indicated. B, CDPK (5 µg) was electrophoresed in a 10 to 15% SDS-gradient polyacrylamide gel, autophosphorylated, and autoradiographed. The Mr's of the labeled bands are indicated.

**Fig. 5.** Phosphorylation of histone H1 by CDPK which had been renatured following SDS-PAGE. CDPK was electrophoresed, renatured, and incubated with histone H1 with no added calcium (lane 3) or with 13 µM-free calcium (lane 4) as described in "Materials and Methods." Histones were also incubated in the protein kinase assay mixture with no CDPK added and with either no added calcium (lane 1) or with 13 µM-free calcium (lane 2). Aliquots of each reaction mixture which contained 20 µg histone H1 were electrophoresed in a 12.5% SDS-polyacrylamide gel. The autoradiogram of the dried gel is shown.
Whether the 46 to 51 kD proteins are different forms of the same protein or different proteins with similar properties remains to be established. Until these proteins can be fully characterized we will refer to them collectively as CDPK since they do retain calcium-dependent activity. The $M_r$ of CDPK calculated from its Stokes radius and its sedimentation coefficient is 59 kD (CP-Evans, MJ Cormier, unpublished observation). This observation and the fact that the 46 to 51 kD proteins are catalytically active indicate that the active kinase is a monomer.

Both the autophosphorylation and histone H1 phosphorylation were stimulated by concentrations of free calcium in the 1 to 10 $\mu$M range (Fig. 2). Although calmodulin-dependent enzymes are also activated by concentrations of free calcium in this range, calmodulin does not confer calcium sensitivity to CDPK. CDPK activity was slightly inhibited by anticalmodulin, but the inhibition was not relieved by the addition of calmodulin (data not shown). CDPK, which had been separated from calmodulin by SDS-PAGE, autophosphorylated in a calcium-dependent manner (Fig. 4), phosphorylated histone H1 in a calcium-dependent manner (Fig. 5) and bound $^{45}$Ca$^{2+}$ when incubated in a buffer which contained 60 mM KCl, 5 mM MgCl$_2$, and micromolar levels of Ca$^{2+}$ (Fig. 6). These data show that CDPK specifically binds calcium, and that calcium directly regulates autophosphorylation as well as the phosphorylation of histone H1. Our results also indicate that phospholipids are not required for the activity of CDPK, because the purified enzyme is active without the addition of phospholipids and any contaminating phospholipids would also be removed from CDPK by SDS-PAGE.

Calmodulin co-purifies with CDPK through DEAE chromatography and TAPP-sepharose chromatography (22). CDPK, like calmodulin, is a high-affinity Ca$^{2+}$-binding protein (Fig. 6) and interacts with W-7 (Fig. 4). Also, the mobility of one of the two CDPK bands in SDS-PAGE was calcium dependent (Fig. 7), as is the mobility of calmodulin (3). These results suggest that the two proteins co-purify because the proteins have similar biochemical properties and not because they are bound to each other. Calmodulin is thought to bind to phenothiazines and naphthalene sulfonamides at a hydrophobic binding site which is exposed when it binds calcium. CDPK binds to TAPP sepharose in the presence of calcium and is eluted from the resin when calcium is removed by EGTA (22). This implies that CDPK also has a hydrophobic binding site that is exposed in the presence of calcium.

Some reports identifying plant calmodulin-dependent protein kinases were based in part on the observation that micromolar levels of calmodulin stimulate protein kinase activity 1.5- to 6-fold (18, 20, 25, 32). Blowers et al. (1) showed that autophosphorylation of a protein kinase, which had been isolated from pea membranes by electrophoresis, was stimulated by the addition of calcium and calmodulin, but they did not show the effect of calmodulin alone. Our results indicate that such observations could be misleading. We have shown that histone H1-phosphorylating activity from all stages of CDPK purification (Table I; Refs. 5, 22) was stimulated a small amount (30-100%) by the addition of micromolar calmodulin regardless of how much endogenous calmodulin is present. Also, CDPK activity was stimulated 75% by micromolar BSA (Table I). Both BSA and calmodulin increased activity in the presence and absence of calcium. These results suggest that the stimulation of CDPK activity by calmodulin is nonspecific.

Another observation that has been relied upon to identify plant calmodulin-dependent protein kinases is the inhibition of protein kinase activity by calmodulin antagonists (18, 20, 25, 32). Our results indicate that these observations could also be misleading since the inhibition of CDPK by the naphthalene sulfonamide W-7 was independent of calmodulin (Fig. 4). W-5, an analog of W-7 that is similar in lipophilicity to W-7 but has lower affinity for calmodulin than W-7 (11), had a higher $IC_{50}$ than W-7 (Fig. 8). The potency of this pair of inhibitors is in the
FIG. 7. The effect of Ca\(^{2+}\) on the mobility of CDPK in SDS-PAGE. Mr markers (lane 1 and 4) and CDPK (5 μg, lanes 2 and 3) were electrophoresed in a 10 to 15% SDS-gradient polyacrylamide gel. Either 2 mM CaCl\(_2\) (+Ca\(^{2+}\)) or 2 mM EGTA was included in the electrophoresis sample buffer. The Mr markers are the same as those listed in the legend to Figure 6.

proper order to indicate that calmodulin could be involved even though we have shown that the effect of W-7 is directly upon the enzyme and is independent of calmodulin. Our data shows that inhibition studies using calmodulin-binding compounds must be interpreted with caution. Phenothiazines and naphthalene sulfonamides have been shown to inhibit other calmodulin-independent protein kinases. Trifluoperazine, a phenothiazine, inhibits phospholipid-dependent kinases with IC\(_{50}\)s ranging from 38 to 50 μM, whereas it inhibits calmodulin-dependent cyclic nucleotide phosphodiesterase with IC\(_{50}\)s of 10 to 12 μM (28). W-7 inhibits phospholipid-dependent, cAMP-dependent, and cGMP-dependent protein kinases with IC\(_{50}\)s in the range of 130 to 360 μM (27, 30) whereas W-7 inhibits the calmodulin-dependent enzymes, myosin light chain kinase and cyclic nucleotide phosphodiesterase, with IC\(_{50}\)s of 50 μM (12) and 26 μM (10), respectively. The IC\(_{50}\) for the inhibition of histone H1-phosphorylation by purified CDPK was 110 μM (Fig. 8) and was 400 μM for less pure CDPK. These values are comparable to the IC\(_{50}\)s reported for the inhibition of the calmodulin-independent protein kinases. Polyai and Micucci (21) have shown that Ca\(^{2+}\)-dependent protein kinases I and II from wheat germ are inhibited by calmodulin antagonists, inhibitors of protein kinase C, and polyamines. These studies point out the difficulty in using calmodulin inhibitors, especially \(\text{in vivo}\) and in crude enzyme preparations.

The stimulation of tonoplast-associated protein kinase activity by nanomolar levels of calmodulin and the complete inhibition of the activity by 1 μM R24571, a calmodulin antagonist, has been reported (31). However, the possibility that the observed \(32^P\)-labeling was due to the incorporation of phosphate into tonoplast membrane ATPase reaction intermediates, rather than to the action of protein kinases, was not eliminated.

Ranjeva \textit{et al.} (23) have deduced, without the use of calmodulin inhibitors, that quinate:NAD\(^+\) 3-oxidoreductase from carrot cell cultures is phosphorylated in a calcium- and calmodulin-dependent manner. That the activity of the quinate:NAD\(^+\) 3-oxidoreductase is apparently regulated by a phosphorylation/dephosphorylation mechanism was indicated by the following observations (23, 24). Quinate:NAD\(^+\) 3-oxidoreductase activity is lost when impure enzyme preparations are incubated with Mg\(^{2+}\), but inclusion of fluoride, a phosphatase inhibitor, in the extract prevents this loss of activity. Addition of ATP to extracts containing inactivated quinate:NAD\(^+\) 3-oxidoreductase restores activity. When extracts containing inactivated quinate:NAD\(^+\) 3-oxidoreductase are first incubated with [\(\gamma\)-\(32^P\)]ATP and then fractionated on an isoelectric focusing column, fractions containing quinate:NAD\(^+\) 3-oxidoreductase activity also contain \(32^P\). However, it was not shown whether inactive enzyme contains phosphate, whether there is a correlation between the degree of \(32^P\)-incorporation and amount of activity, or whether the \(32^P\) associated with the active enzyme could subsequently be removed with a concomitant loss of activity. In more recent experiments, it was shown that calcium and calmodulin are required for the reactivation process (23). If either calcium is removed from the extract by chelation with EGTA or calmodulin...
is removed by chromatography on an affinity column, quin-ate:NAD+ 3-oxidoreductase activity is not restored by the addition of ATP. When 0.5 μM bovine brain calmodulin and 1 mM CaCl₂ are added to the inactive calmodulin-depleted extract, 130% of the original activity is restored. It would be very interesting if the calcium- and calmodulin-dependent protein kinase implicated in these studies can be isolated and characterized. Besides its requirement for calmodulin, this protein kinase is unlike CDPK in that it does not bind to either DEAE-Sepahcel (24) or to a calmodulin affinity resin (23).

Our results show that CDPK binds calcium directly, is activated 100-fold by micromolar calcium, is not dependent on calmodulin or phospholipid for activity, and is directly inhibited by W-7. The calcium-dependent protein kinases discovered to date have been dependent on either calmodulin or phospholipids for activity. Therefore, CDPK represents a new class of calcium dependent protein kinase.

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