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Localization, Solubilization and Characterization of Plant Membrane-Associated Calcium-Dependent Protein Kinases[1]

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ABSTRACT

Membrane fractions from mature silver beet (*Beta vulgaris*) deveined leaf and leaf stem homogenates have associated Ca^{2+}-dependent protein kinase. The Ca^{2+}-dependent protein kinase activity is associated with plasma membranes (density 1.14–1.18 grams per cubic centimeter) as determined from copurification on isopycnic centrifugation with plasma membrane markers such as β-glucan synthetase, eosin-5-maleimide-labeling, and specific naphthylphthalamic acid-binding. The Ca^{2+}-dependent protein kinase is not specifically associated with chloroplasts or mitochondria. The membrane-bound Ca^{2+}-dependent protein kinases were solubilized with 0.8% (volume/volume) Nonidet P40. The solubilized enzymes were extensively purified by a protocol involving binding to diethylaminoethyl-cellulose (Whatman DE-52), Ca^{2+}-dependent binding to phenyl-Sepharose CL-4B, gradient elution from diethylaminoethyl-Sephacel (resolving two distinct Ca^{2+}-dependent protein kinases), and gel filtration on Ultrogel AcA 44. These two membrane-derived enzymes have similar molecular weights but differ in protein substrate specificity, in K_m values for ATP, and in Ca^{2+}-independent activation by unsaturated fatty acids. The membrane-bound enzymes correspond closely in these properties to two Ca^{2+}-dependent protein kinases present in the soluble phase.

Protein phosphorylation catalyzed by CDPKs[2] represents a major mechanism for stimulus-response coupling in both animal (13) and plant systems (7, 18, 19). CDPK has been described in a variety of soluble and particulate preparations from plants (for review, see Refs. 7, 18, and 19). Soluble CDPKs have been extensively purified from several plant sources (5, 11, 16). Some soluble and particulate CDPK preparations have been shown to be activated to varying degrees by certain lipids (for references, see Ref. 16) as is animal protein kinase C (13). Plant CDPKs can be activated by certain unsaturated fatty acids (10, 11) as are different forms of animal protein kinase C (12, 22).

Pea membrane-associated CDPK (2, 8) may be associated with the plasma membrane on the basis of enhanced activity in plasma-membrane-enriched fractions and the occurrence of a CDPK peak at an appropriate density (ρ = 1.14 g cm^{-3}) on isopycnic centrifugation (8). CDPK is associated with highly purified *Lolium* endosperm plasma membrane preparations (17) but is also present associated with other membrane fractions. We have extensively purified and characterized two soluble CDPKs (enzymes I and II) from silver beet leaves. However, a substantial proportion of CDPK activity in silver beet leaves is membrane-associated (16). The present paper describes the subcellular localization of this particulate CDPK activity and the solubilization, extensive purification, and characterization of two distinct membrane-associated CDPKs from silver beet leaves.

MATERIALS AND METHODS

Plant Material and Chemicals. Mature leaves of silver beet (*Beta vulgaris*) were purchased locally. [γ-^{32}P]ATP (3 Ci/mmol) was obtained from Amersham International, UK. Calf thymus histone (catalog specification: III-S), dephosphorylated casein, ATP, BSA, enzymes for gel filtration column calibration, and fatty acids were obtained from the Sigma Chemical Co., St. Louis, MO. Fatty acids were dissolved in H_2O and neutralized to yield clear 2.0 mM solutions. Ultrogel AcA 44 was obtained from LKB Produkter, DE-52 from Whatman, and DEAE-Sephacel and phenyl-Sepharose CL-4B from Pharmacia. [2,3,4,5-^{3}H]NPA (specific activity 55 Ci/mmol) was obtained from Commissariat à L'Energie Atomique, Department de Biologie, Gif-sur-Yvette, France, and unlabeled NPA from T.C.I., Japan. EMA was obtained from Molecular Probes Inc., Eugene, OR.

Homogenization and Differential Centrifugation. All operations were performed at 0 to 4°C. Two types of leaf tissue were extracted in separate experiments, namely non-green leaf stem tissue and deveined, green leaf tissue. Plant tissue was juiced with a Chop-Rite juicer into a chilled collecting vessel to yield a homogenate containing about 1 g (wet weight) plant material/mL in 0.25 M sucrose, 0.15 M Tricine (pH 7.4), 0.01 M KCl, 1 mM $MgCl_2$, 1 mM EDTA, 0.5 mM PMSF, 0.25% (v/v) ethanol, and 10 mM 2-mercaptoethanol. The homogenate was filtered through Miracloth and centrifuged at 250*g* for 5 min. Deveined leaf homogenates (but not chloroplast-free leaf stem homogenates) were routinely depleted of almost half of the chloroplast fraction (as determined from Chl content) by centrifugation at 3,000*g* for 1 min. A 15,000*g* pellet fraction or a high-speed pellet fraction was prepared by centrifugation of the chloroplast-depleted homogenate at 15,000*g* for 15 min or 156,000*g* for 30 min, respectively. Corresponding particulate fractions were obtained in the same way from leaf stem homogenates.

Density Gradient Centrifugation. Gradients of 11 to 52% (w/w) sucrose in 1 mM EDTA and 1 mM $MgCl_2$ (pH 7.2) were generated using an ISCO gradient maker and underlaid with 3 ml 63% (w/w) sucrose. A 2- to 3-mL sample was layered on top of the gradient and overlaid with 2 mL 50 mM Tris (Cl^-, pH 7.4) and 10 mM 2-mercaptoethanol. Sucrose density gradients were centrifuged at 132,000*g* for 2.5 h in a Beckman SW27 swing-out rotor. Gradients were fractionated into 1.2-mL fractions using an ISCO density gradient fractionator. Sucrose concentrations (% w/w) were determined using an Atago refractometer.

Fluorescent Labeling of Membranes. Mature silver beet leaves were labeled with EMA by standing the stems in 75 μM EMA for 48 h at room temperature. Subsequent treatment of stem tissue

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[2] Abbreviations: CDPK, Ca^{2+}-dependent protein kinase; EMA, eosin-5-maleimide; NPA, naphthylphthalamic acid; PMSF, phenylmethylsulfonyl fluoride.

was derived from Spring and Krauss (23). Stems were removed, cut ends deleted, and the tissue washed successively at 0° in H_2O and in a plasmolyzing solution containing 0.4 M sucrose, 10 mM reduced glutathione, and 10 mM potassium phosphate (pH 7.5). The tissue was homogenized in a Chop-Rite juicer to yield 1 g fresh wt tissue/mL homogenate in 0.3 M sucrose, 10 mM EGTA, 10 mM reduced glutathione, 50 mM potassium phosphate (pH 7.5), 0.5 mM PMSF, and 0.25% (w/v) ethanol. The homogenate was filtered through Miracloth, centrifuged at 250*g* for 5 min, and then a low speed pellet was obtained by centrifugation at 15,000*g* for 15 min. The pellet was resuspended by hand in a Potter-Elvehjem homogenizer in 0.3 M sucrose, 1 mM EGTA, and 10 mM phosphate (Na^+, pH 7.5) and was analyzed by sucrose density gradient centrifugation. EMA labeling of plant fractions was determined by fluorescence measurements (excitation λ: 518 nm; emission λ: 540 nm) using a Hitachi Model 650-10S fluorescence spectrophotometer.

NPA Binding. NPA binding to sucrose density gradient fractions was determined in duplicate using [2,3,4,5-^{3}H]NPA as described by Thomson *et al.* (24). Binding was performed in 1.5 mL polycarbonate tubes which were centrifuged at *ca.* 5×10^5 g for 5 min in a Beckman TL-100 centrifuge. Bound [2,3,4,5-^{3}H]NPA was extracted from the pellets by overnight suspension in absolute ethanol and counted using an H_2O-compatible scintillation cocktail (15). Specific NPA binding was determined from the amount of [2,3,4,5-^{3}H]NPA displaced by 10^{-5} M NPA in the binding assay (24).

Enzyme, Chl, and Protein Assays. All enzyme assays were conducted in duplicate. Cyt *c* oxidase was measured as described by Hodges and Leonard (9) and β-glucan synthetase (with 1 mM UDP-glucose in the assay) by the method of Henry *et al.* (6). Chl was determined as described by Barra and Crane (1). Protein was determined by the method of Sedmak and Grossberg (21) using crystalline BSA as a standard. Protein kinase was routinely assayed radiochemically (16) at 30°C in a standard reaction medium (100 μL final volume) containing 62.5 mM Tris (Cl^-, pH 8.0), 10 mM $MgCl_2$, 10 mM DTT, 0.25 mM EGTA, 1.25 mM $CaCl_2$, 2.5 mM 2-mercaptoethanol, 50 mM NaCl, 25 μM ATP (specific activity of [γ-^{32}P]ATP: about 100 mCi/mmol), and 1 mg/mL lysine-rich histone (Sigma Chemical Co. catalog specification: type III-S). Assay ^{32}P-incorporation values were corrected by subtraction of appropriate control values determined in the absence of added protein kinase.

Partial Purification of Leaf Particulate Ca^{2+}-Dependent Protein Kinases. All procedures were conducted at 0 to 4°C. One kilogram of deveined mature silver beet leaves was homogenized and centrifuged as described above to yield a high speed pellet fraction. This fraction was resuspended in 400 mL homogenizing buffer and recentrifuged at 156,000*g* for 30 min to remove residual soluble material. The washed, high-speed pellet was extracted twice with 0.8% (v/v) Nonidet P40 in buffer A (50 mM Tris [Cl^-, pH 8.0]-10 mM 2-mercaptoethanol). The combined detergent extracts were subjected to a protocol based on that employed to extensively purify two soluble CDPKs from silver beet leaves (16). This procedure involved batchwise absorption to 150 g DEAE-cellulose (Whatman DE-52) (the protein kinase being eluted by 0.5 M NaCl-buffer A), absorption to 100 ml (bed volume) phenyl-Sepharose CL-4B in 0.5 M NaCl-buffer A-1 mM $CaCl_2$ (the protein kinase being eluted by buffer A-1 mM EGTA after extensive washing of the matrix with buffer A-1 mM $CaCl_2$), and elution from DEAE-Sephacel (7 $cm^2 \times 11$ cm) by a linear gradient of NaCl concentration in buffer A to separate two CDPK enzymes (denoted PI and PII). CDPKs PI and PII were finally subjected to gel filtration on an Ultrogel AcA 44 column (6 $cm^2 \times 45$ cm) in 0.2 M NaCl-buffer A. Soluble CDPKs (denoted SI and SII) were extensively purified from the high speed supernatant fraction from silver beet leaf extracts as previously described (16).

RESULTS AND DISCUSSION

Localization of Membrane-Associated CDPK. After high speed centrifugation (156,000*g* for 30 min) of silver beet leaf homogenates, about 30% of Ca^{2+}-dependent histone kinase activity is associated with the washed particulate fraction. Ca^{2+}-dependent histone kinase was determined by conducting assays in the standard reaction conditions in the presence of Ca^{2+} (0.25 mM EGTA and 1.25 mM $CaCl_2$ present) or in the absence of Ca^{2+} (0.25 mM EGTA and no added $CaCl_2$ present). After sucrose density gradient analysis of leaf membranes or homogenates, it was found that Ca^{2+}-independent histone kinase was less than 5% of the total activity measured in the presence of added Ca^{2+}. It should be noted that in all sucrose density gradient analyses, fractions were diluted prior to protein kinase assays to minimize the effect of endogenous protein kinase reaction inhibitors. Fractions at the top of gradients containing soluble material were routinely diluted 20-fold and membrane fractions 5-fold.

Sucrose density gradient centrifugation of a deveined leaf homogenate (after prior removal of about 50% of the chloroplasts) yields a CDPK peak at 38.5% (w/w) sucrose ($\rho = 1.169$ g cm^{-3}) and a CDPK shoulder at 34% (w/w) sucrose ($\rho = 1.146$ g cm^{-3}) in addition to a major peak of CDPK in the soluble zone (Fig. 1). The peak of membrane-associated CDPK does not correspond to the peak of Cyt *c* oxidase (a marker for mitochondria) which occurs at 40% (w/w) sucrose ($\rho = 1.177$ g cm^{-3}) (Fig. 1). The CDPK peak is coincident with the Chl peak (Fig. 1). However, as shown below, CDPK is not associated with chloroplasts.

Gradient centrifugation of the leaf chloroplast fraction (from centrifugation of the homogenate at 3,000*g* for 1 min) yields a Chl peak at 38% (w/w) sucrose ($\rho = 1.166$ g cm^{-3}) but with a low amount of associated CDPK (peak CDPK/Chl ratio: 0.4 pmol $min^{-1} \cdot \mu g\ Chl^{-1}$) (Fig. 2A). In contrast, gradient centrifugation of the pelleted fraction deriving from the subsequent centrifugation of the chloroplast-depleted homogenate at 15,000*g* for 15 min yields quite distinct CDPK and Chl profiles (Fig. 2B), indicating that the CDPK is not specifically associated with chloroplasts. CDPK and Chl peak at 37% (w/w) sucrose ($\rho = 1.160$ g cm^{-3}) and 38.5% (w/w) sucrose ($\rho = 1.169$ g cm^{-3}), respectively. At the CDPK peak, the CDPK/Chl ratio is 10 pmol $min^{-1} \cdot \mu g \cdot Chl^{-1}$ (Fig. 2B), 25 times the CDPK/Chl ratio found for the Chl peak from density gradient centrifugation of the chloroplast-enriched 3,000*g* pellet (Fig. 2A). This argues against a specific chloroplast localization of CDPK. The lack of correspondence of CDPK and Cyt *c* oxidase profiles and peaks (Fig. 2B) provides further evidence against a mitochondrial localization of CDPK (*cf.* Figs. 1 and 2B).

Further evidence that CDPK is not specifically associated with mitochondria or chloroplasts derives from sucrose density gradient centrifugation of leaf stem homogenates which contain no chloroplasts (Fig. 3). The CDPK and Cyt *c* oxidase profiles are quite distinct, with maximal activities at 34% (w/w) sucrose ($\rho = 1.146$ g cm^{-3}) and 37.5% (w/w) sucrose ($\rho = 1.163$ g cm^{-3}), respectively (Fig. 3).

At this stage of the analysis, we concluded that the CDPK is not specifically localized on chloroplast or mitochondrial membranes. In view of the equilibrium densities of the CDPK-bearing membranes ($\rho = 1.146$–1.169 g cm^{-3}), localization of CDPK on plasma membranes (peak banding densities in the range $\rho = 1.13$–1.18 g cm^{-3}) (3, 20) seemed likely. Accordingly, we examined the distribution of CDPK and some plasma membrane markers on density gradient centrifugation of stem and deveined leaf membranes. Density gradient centrifugation of the resuspended high speed pellet (156,000*g* for 30 min) from leaf stem

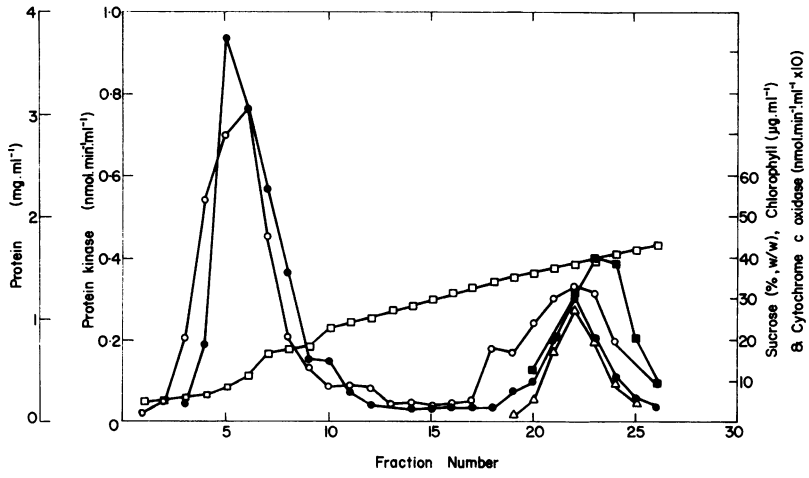

FIG. 1. Sucrose density gradient centrifugation of a deveined leaf homogenate after chloroplast depletion. A deveined leaf homogenate was centrifuged at 3,000*g* for 1 min to partially remove chloroplasts, and a sample of the supernatant (deriving from the equivalent of 2.2 g fresh weight tissue) was analyzed by sucrose density gradient centrifugation as described in "Materials and Methods." ●——●, protein concentration (mg mL^{-1}); ○——○ protein kinase (nmol $min^{-1} \cdot mL^{-1}$), △——△, Chl concentration (μg mL^{-1}); □——□, sucrose concentration (%, w/w); ■——■, Cyt *c* oxidase (nmol min^{-1} mL^{-1}).

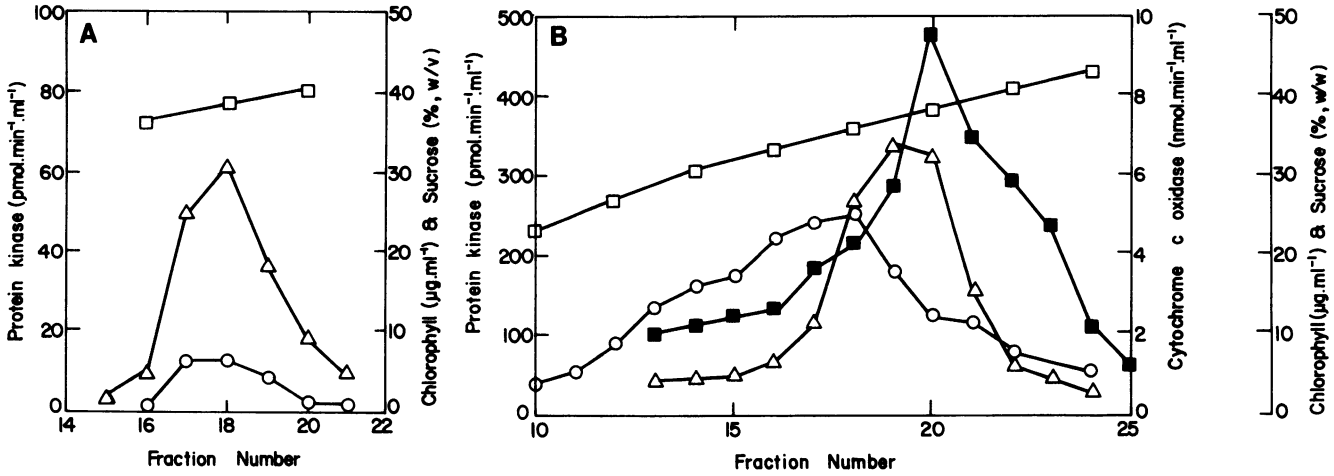

FIG. 2. Sucrose density gradient centrifugation of material pelleted from a deveined leaf homogenate. The pelleted material examined was the chloroplast fraction pelleted at 3,000*g* for 1 min (A) and the fraction pelleted subsequently at 15,000*g* for 15 min (B). The material applied in (A) and (B) was derived from the equivalent of 23 g and 6 g fresh weight of tissue, respectively. ○——○, protein kinase (pmol $min^{-1} \cdot mL^{-1}$); △——△, Chl concentration (μg ml^{-1}); □——□, sucrose concentration (%, w/w); ■——■, Cyt *c* oxidase (nmol $min^{-1} \cdot mL^{-1}$).

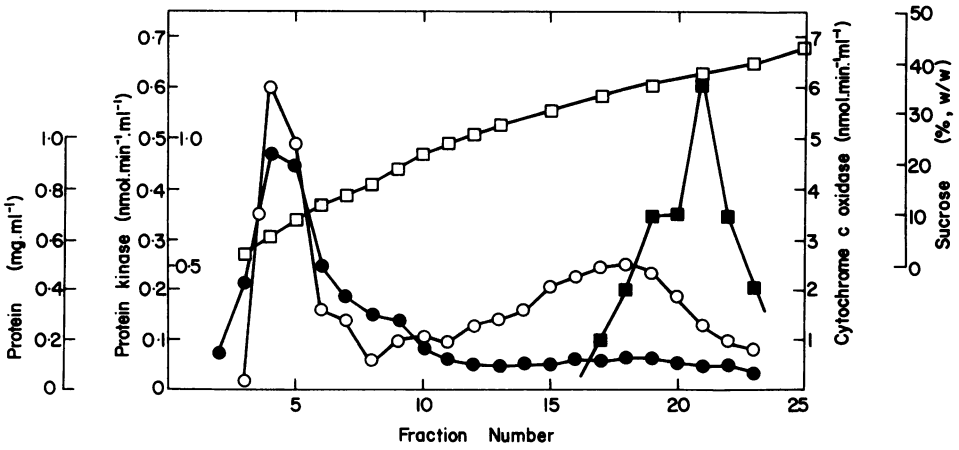

FIG. 3. Sucrose density gradient centrifugation of a stem homogenate. A sample of stem homogenate (deriving from the equivalent of 3.0 g fresh weight of tissue) was analyzed. ○——○, protein kinase (nmol min^{-1} mL^{-1}); ●——●, protein concentration; □——□, sucrose concentration (%, w/w); ■——■, Cyt *c* oxidase (nmol min^{-1} mL^{-1}).

homogenates yields a broad bimodal distribution of CDPK with peaks at 32% (w/w) sucrose (ρ = 1.137 g cm^{-3}) and 36% (w/w) sucrose (ρ = 1.156 g cm^{-3}) (Fig. 4). Specific [2,3,4,5-^{3}H]NPA binding similarly occurs in a broad zone with the peak at 35.5% (w/w) sucrose (ρ = 1.154 g cm^{-3}). However, Cyt *c* oxidase occurs in a relatively sharp zone, with peak activity at 34.5% (w/w) sucrose (ρ = 1.148 g cm^{-3}) (Fig. 4). This suggests that CDPK is associated with membranes carrying specific NPA binding sites, and there is considerable evidence that such sites are associated with plant plasma membranes (3).

Density gradient centrifugation of a leaf stem homogenate demonstrates that the profiles and peaks of both CDPK and β-glucan synthetase are coincident (Fig. 5). The peaks of both activities occur at 35% (w/w) sucrose (ρ = 1.151 g cm^{-3}). There

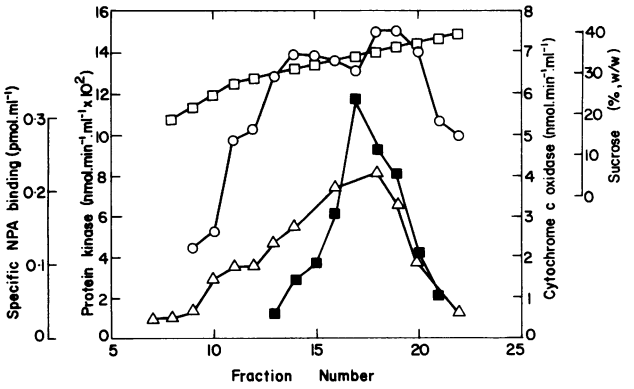

FIG. 4. Sucrose density gradient centrifugation of a high-speed pellet from a stem homogenate. Material from high-speed centrifugation (156,800g for 30 min) of a stem homogenate and deriving from the equivalent of 18 g fresh weight of tissue was analyzed. O——O, protein kinase (nmol min^{-1} mL^{-1}); Δ——Δ, specific NPA binding (pmol ml^{-1}); ■——■, Cyt c oxidase (nmol min^{-1} mL^{-1}).

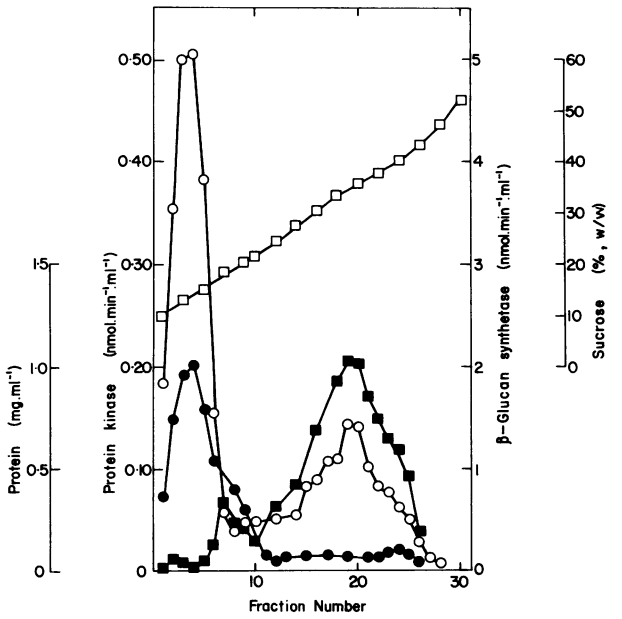

FIG. 5. Sucrose density gradient centrifugation of a leaf stem homogenate. A stem homogenate (from the equivalent of 3.0 g fresh weight of starting material) was analyzed as described in "Materials and Methods." □——□, sucrose concentration (%, w/w); O——O, protein kinase (nmol $min^{-1}\cdot mL^{-1}$); ●——●, protein concentration (mg mL^{-1}); ■——■, β-glucan synthetase (nmol glucose incorporated $min^{-1}\cdot mL^{-1}$).

is much evidence for the association of β-glucan synthetase with plasma membranes (3, 6), and, accordingly, this result further suggests that particulate CDPK is associated with plasma membranes.

Density gradient centrifugation of the 15,000g pellet fraction (15,000g for 5 min) from the stem homogenate results in CDPK in the soluble zone and two zones of membrane-associated CDPK peaking at 32% (w/w) and 39% (w/w) sucrose (ρ = 1.137 and 1.172 g cm^{-3}, respectively) (Fig. 6). Two β-glucan synthetase peaks also occur at approximately the same positions, namely at 32% (w/w) and 39.5% (w/w) sucrose (ρ = 1.137 and 1.174 g cm^{-3}, respectively) (Fig. 6). EMA fluorescence is a marker for membranes exposed to EMA in the intact tissue before reaction of excess EMA with reduced glutathione and homogenizing of the tissue. EMA fluorescence is a marker for the plasma membrane since EMA is a membrane-impermeant sulfhydryl reagent (23). Membrane-associated EMA fluorescence occurs in two peaks banding close to the CDPK and β-glucan synthetase peaks, namely at 34% (w/w) and 39.5% (w/w) (ρ = 1.146 and 1.174 g cm^{-3}) (Fig. 6). This provides further evidence for the association of CDPK and β-glucan synthetase with plasma membranes. It should be noted that dual peaks of CDPK in the plasma membrane region were also observed on density gradient centrifugation of the high speed pellet from stem homogenates (Fig. 4), whereas with analysis of unpelleted membranes in the stem homogenate, only one peak of CDPK is observed (Fig. 3).

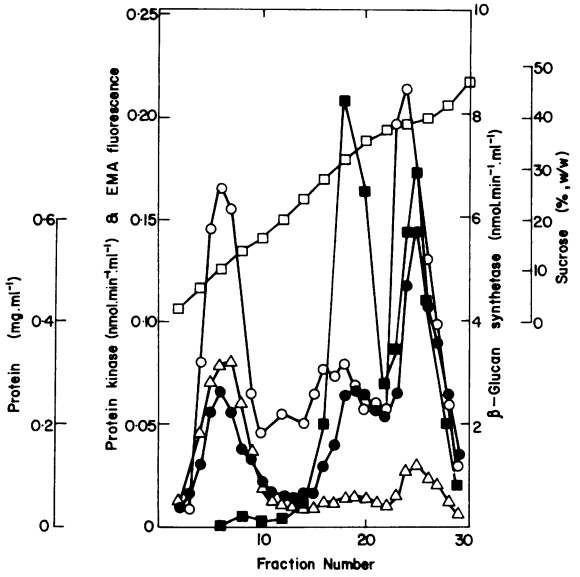

FIG. 6. Sucrose density gradient centrifugation of the large granular fraction from a stem homogenate. The large granular fraction was pelleted from the stem homogenate by centrifugation at 15,000g for 15 min as described in "Materials and Methods." Material derived from the equivalent of 30 g fresh weight starting tissue was applied. O——O, protein kinase (nmol $min^{-1}\cdot mL^{-1}$); ●——●, protein concentration (mg ml^{-1}), □——□, sucrose concentration (%, w/w); ■——■, β-glucan synthetase (nmol $min^{-1}\cdot mL^{-1}$); Δ——Δ, EMA fluorescence (relative units).

The correspondence between CDPK and β-glucan synthetase profiles and peaks found on density gradient analysis of leaf stem homogenates and pelleted membrane fractions (Figs. 5 and 6) is also observed on such analysis of the deveined leaf homogenate (Fig. 7). CDPK and β-glucan synthetase profiles are coincident and peak at the same position, namely 38% (w/w) sucrose (ρ = 1.166 g cm^{-3}). We conclude that CDPK is associated with plasma membranes in both the chloroplast-containing leaf tissue as well as in the non-green stem tissue of mature silver beet leaves.

Solubilization and Partial Purification of Particulate CDPKs. CDPK associated with the washed high speed pellet can be largely (about 90%) solubilized by 0.8% (v/v) Nonidet P40 in buffer A. In contrast, the CDPK activity is not solubilized by repeated extraction with 1 mM EGTA-buffer A. The solubilized CDPK was extensively purified by application of a protocol involving batchwise elution from DEAE-cellulose (Whatman DE-52), Ca^{2+}-dependent hydrophobic chromatography on phenyl Sepharose CL-4B, gradient elution from DEAE-Sephacel (Fig. 8), and gel filtration on Ultrogel AcA 44. This procedure is essentially that devised for the extensive purification of two CDPKs (denoted enzymes I and II) from the high-speed supernatant fraction from mature silver beet leaf homogenates (16). As found for the soluble CDPK activity (16), chromatography on DEAE-Sephacel resolves solubilized particulate CDPK into two fractions (denoted PI and PII) eluting at 0.18 M NaCl-buffer A and 0.28 M

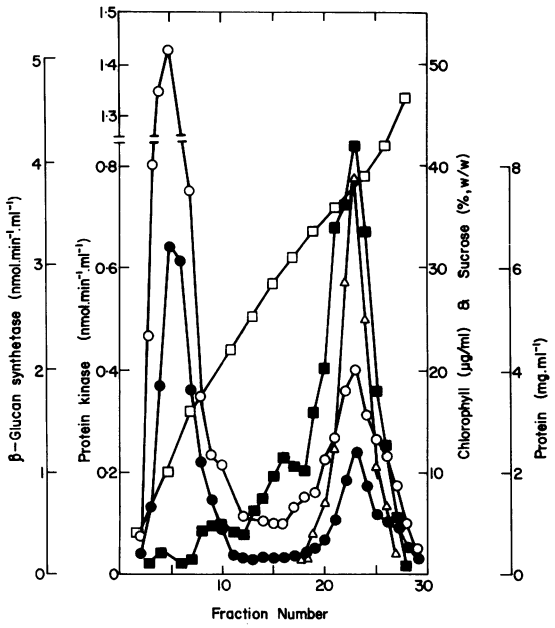

FIG. 7. Sucrose density gradient centrifugation of a deveined leaf homogenate. The homogenate applied was derived from the equivalent of 3.7 g fresh weight deveined leaf tissue. □——□, sucrose concentration (%, w/w); ○——○, protein kinase (nmol $min^{-1} \cdot mL^{-1}$); ●——●, protein concentration (mg mL^{-1}); △——△, Chl concentration (μg ml^{-1}), ■——■, β-glucan synthetase (nmol glucose incorporated $min^{-1} \cdot mL^{-1}$).

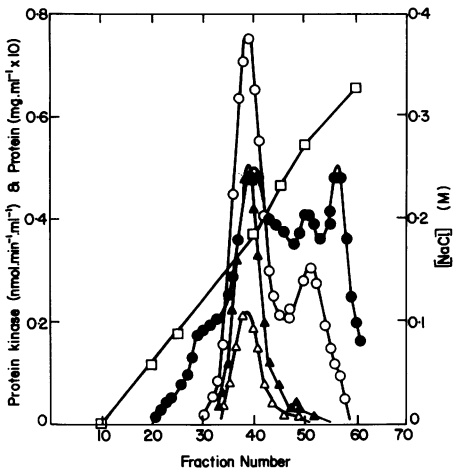

FIG. 8. Elution of CDPKs PI and PII from DEAE-Sephacel by a NaCl concentration gradient in buffer A. Protein kinase activity (nmol $min^{-1} \cdot mL^{-1}$) was measured using the following protein substrates (at 1 mg/ml in the reactions): histone type III-S (○——○); BSA (▲——▲); dephosphorylated casein (△——△). ●——●, protein concentration; □——□, NaCl concentration.

NaCl-buffer A, respectively (Fig. 8). Enzymes PI and PII differ in substrate specificity. With 1 mg/ml histone (Sigma Chemical Co. type III-S), BSA, or casein as substrates, rates of phosphorylation (relative to the rate of histone phosphorylation) with PI were 1.0, 0.7, and 0.3, respectively, and with PII, 1.0, 0.1, and 0.01, respectively (Fig. 8). The same pattern of elution and differential substrate specificity is observed on elution of soluble enzymes I and II from DEAE-Sephacel (16). However, while soluble enzymes I and II contribute 27 ± 9% and 73 ± 9% (mean ± SD from three determinations), respectively, of total CDPK activity (measured with histone as substrate) resolved on DEAE-Sephacel, enzymes PI and PII contribute 63 ± 9% and 37 ± 9% (mean ± SD from four determinations), respectively, of membrane-derived CDPK resolved at this stage.

Gel filtration of PI and PII on Ultrogel AcA 44 in 0.2 M NaCl-buffer A yields single peaks of CDPK and does not alter the ratio of protein kinase activities measured with histone, BSA, or casein as substrates (data not shown). Molecular weight estimates were made from gel filtration on an Ultrogel AcA44 column calibrated with proteins of known mol wt. The mol wts are 53,000 ± 4,000 and 51,000 ± 1000 (means ± SDs from four determinations) for enzymes PI and PII, respectively. These are similar to estimates of 56,000 and 57,000 for soluble enzymes I and II (16). It should be noted that while detergent is required for the solubilization of the CDPKs, it is not subsequently required for enzyme stability or activity. The behavior of extensively purified PI and PII as relatively low molecular weight entities on gel filtration indicates that these enzymes do not form high mol wt aggregates in the absence of detergent.

The overall purification process results in a purification of 230-fold and 74-fold for PI and PII, respectively, relative to the solubilized CDPK (Table I). The overall yield is only 4% and 2% for enzymes PI and PII, respectively. A major loss of activity occurs at the stage of the final gel filtration involving only about 1 mg of protein in either enzyme preparation (Table I). This loss may derive from the hydrophobic nature of these proteins and the small amounts of protein involved at this stage of the purification process. The specific activities of PI and PII preparations (Table I) are similar to those found for partially purified preparations of soluble enzymes I and II (16). None of these soluble (16) or membrane-derived preparations are homogeneous on the basis of SDS-PAGE, and the specific activities (16) (Table I) are about 100 times lower than those of preparations of pure protein kinase C (13).

Comparison of Properties of Membrane-Derived and Soluble Leaf CDPKs. As shown above, membrane-derived CDPKs PI and PII closely resemble the previously described soluble CDPKs I and II in terms of purification behavior, molecular size, and differential protein substrate specificity. Like soluble enzymes I and II, PI and PII both phosphorylate a synthetic peptide, $S6_{229-239}$ (corresponding to the 229–239 amino acid sequence of rat ribosomal protein S6) (25) with K_m values of about 0.1 mM. The K_m values for BSA are 0.06 mM for both enzymes PI and PII, similar to the values found for enzymes I and II (16). The K_m values for histone (type III-S) are 1.0 ± 0.3 mg/ml and 0.7 ± 0.2 mg/ml for enzymes PI and PII, respectively, similar to the values found for soluble enzymes I and II (16). As found for soluble enzymes I and II (16), while the K_m values for both histone (type III-S) and BSA are similar for PI and PII, the rates of phosphorylation with 1 mg/ml BSA as compared to histone

Table I. *Partial Purification of Solubilized CDPK Enzymes PI and PII*

A representative purification schedule is presented. CDPK enzymes PI and PII were partially purified with 980 g mature silver beet leaves as the starting material. CDPK was determined in the standard reaction conditions with 1 mg/ml histone (type III-S) as substrate.

Purification Stage	Protein	Total CDPK Activity	CDPK Specific Activity
	mg	*nmol min⁻¹*	*nmol min⁻¹ · mg protein⁻¹*
Nonidet P40 extract	3700	386	0.10
DE-52	535	190	0.36
Phenyl-Sepharose CL-4B	10.4	148	14.2
DEAE-Sephacel			
PI	5.0	56	11.2
PII	3.1	20	6.5
Ultrogel AcA44			
PI	0.6	14	23
PII	1.0	7.4	7.4

(type III-S) as a substrate are much lower for PII than for PI (Fig. 8).

The K_m values for ATP for enzymes PI and PII are significantly different. In an experiment involving PI and PII preparations made at the same time and employing exactly the same reagents, the K_m values for ATP were 37.0 ± 5.1 μM and 20.0 ± 2.3 μM (mean ± SEM) for PI and PII, respectively. K_m values were determined here by fitting kinetic data (rates of histone phosphorylation at various ATP concentrations) to the Michaelis-Menten equation using a nonlinear, least-squares, curve-fitting program (4). In a second experiment using PI and PII preparations from a second overall preparative sequence, K_m values for ATP (determined as described above) were 37.5 ± 5.3 μM and 13.1 ± 1.7 μM for PI and PII, respectively. We have shown that soluble CDPK enzymes I and II also have significantly different K_m values for ATP (25 and 12 μM, respectively) (16).

We have previously shown (10) that in the presence of 20% (v/v) dimethylsulfoxide, soluble enzyme II is activated to a much greater degree than enzyme I by arachidonic acid and other unsaturated fatty acids in the absence of Ca^{2+}. The same differential activation of enzyme II (as compared to enzyme I) is also observed when dimethylsulfoxide is omitted from the reaction (Table II). Membrane-derived enzyme PII is similarly activated to a much greater degree than PI by arachidonate in the absence of Ca^{2+} (Fig. 9) and by oleate and linoleate in the same conditions (Table II). It should be noted that all three unsaturated fatty acids inhibit Ca^{2+}-activated PI (and I) activity to a greater degree than the activity of PII (or II) in the presence of Ca^{2+} (Fig. 9, Table II). It is most unlikely that these differential effects derive from a protein contaminant in the enzyme preparations used, given the different sources (soluble or particulate) of the preparations, the high degree of purity of the preparations (16) (Table I), and the very small amount of preparation protein (about 1 μg) added to the assays (*cf.* Tables I, II, and Fig. 9). Similar effects have been observed with even higher purity CDPK from wheat germ (11).

General Discussion. The membrane-associated CDPK from deveined silver beet leaf and leaf stem tissue comigrates with several plasma membrane markers, namely β-glucan synthetase and specific NPA binding on sucrose density gradient centrifugation (Figs. 4–7). Evidence for exclusive localization of β-glucan synthetase and specific NPA binding on plasma membranes is not unequivocal due in part to the possible heterogeneity of plasma membrane-derived fragments and a consequent broad distribution of such components on isopycnic centrifugation analysis (3). However, in the present study, use has also been made of the impermeant reagent EMA for the fluorescent tagging of plasma membranes. The co-migration of CDPK with EMA labeling (Fig. 6) provides further evidence for an association of CDPK with plasma membranes. In addition, the density range in which maximal CDPK binding occurs (ρ = 1.14–1.18 g cm^{-3}) is within the range reported for peak equilibrium densities of plant plasma membrane fragments (ρ = 1.13–1.18 g cm^{-3}) (3, 20). Hetherington and Trewavas (8) similarly found CDPK components with peak densities of about 1.14 and 1.16 g cm^{-3} on isopycnic centrifugation of pea membranes. We conclude that the peak membrane-associated silver beet leaf CDPK is associated with plasma membranes. Golgi apparatus and endoplasmic reticulum membranes equilibrate at peak densities of approximately 1.12 to 1.14 g cm^{-3} (3) and the tonoplast at a peak density of approximately 1.10 to 1.12 g cm^{-3} (3, 20). Thus, while such membranes may well contaminate CDPK fractions after isopycnic centrifugation, the peak CDPK activity bands at higher densities (1.14–1.18 g cm^{-3}). While cross-contamination of particle zones cannot be prevented in these separations, it is unlikely that Golgi, endoplasmic reticulum, or tonoplast membranes constitute anything but a minor contamination in the approximately 1.17 g cm^{-3} (39% [w/w] sucrose) density region of maximal CDPK activity observed in some profiles (Figs. 6 and 7).

A plasma membrane location for the CDPK activity is consistent with the nature of the Ca^{2+}-dependence of these enzymes. Preparations of soluble enzymes I and II and of membrane-derived enzymes PI and PII are largely dependent upon Ca^{2+} for activity and are maximally activated by free Ca^{2+} in the range 10^{-6} to 10^{-5} M (16) (Fig. 9). These activating, albeit low, free Ca^{2+} concentrations are found in the cytosol of electrically stimulated plant cells (7). Furthermore, in the absence of positive effectors such as unsaturated fatty acids, these CDPKs are relatively inactive in the presence of free Ca^{2+} concentrations of about 10^{-7} M found in the cytosol of unstimulated plant cells (7, 16) (Fig. 9).

Membrane-derived CDPK enzymes PI and PII are very similar in many properties to the corresponding enzymes I and II that have been extensively purified from the high-speed supernatant fraction of silver beet leaf homogenates. Such properties include purification behavior on DEAE-Sephacel, molecular size and differential protein substrate specificities, K_m values for ATP, and patterns of activation by unsaturated fatty acids in the absence of Ca^{2+}. It is likely that PI and PII are simply soluble enzymes I and II that have become membrane associated (before or after extraction) or forms of these enzymes—deriving from slight covalent modification (including proteolysis) or ligand binding—that have become membrane associated. Alternatively, it is possible that the soluble enzymes derive from specific proteolysis of the membrane-bound enzymes. Resolution of these possibilities must await purification of these proteins, immunolocalization studies, and structural comparisons of the pure proteins. It should be noted that the association of the CDPKs with membranes is not simply Ca^{2+}-dependent hydrophobic association (of the kind exploited in partial purification of these enzymes), since the CDPKs are not removed from the membranes by repeated extraction with 1 mM EGTA-buffer A.

The silver beet membrane-derived and soluble (16) CDPKs are similar to CDPKs extensively purified from soybean (5) and wheat germ (11) in purification properties, Ca^{2+}-dependence, and substrate specificity but have lower apparent mol wts (16). Silver beet leaf CDPKs are similar to animal protein kinase C in many properties including substrate specificity, Ca^{2+}-dependence, Ca^{2+}-dependent binding to hydrophobic ligands, inhibition by a variety of calmodulin antagonists (16), and activation by

Table II. *Ca^{2+}-Independent Activation of CDPK Enzymes PI, PII, I, and II by Unsaturated fatty Acids*

CDPK was measured in the standard assay conditions with 0.8 mg/ml histone (type III-S) as substrate. Assays were conducted in the presence of 0.2 mM EGTA and no added $CaCl_2$ ($-Ca^{2+}$) or in the presence of 0.2 mM EGTA and 0.24 mM $CaCl_2$ ($+Ca^{2+}$). Fatty acids (clear, neutralized, aqueous 2.0 mM solutions) were added to a final concentration of 0.4 mM. Rates of phosphorylation are expressed as percent of control rates measured in the presence of Ca^{2+} (11, 14, 18, and 17 pmol min^{-1} for PI, PII, I, and II, respectively.

Addition	Protein Kinase			
	PI	PII	I	II
	% control			
None ($-Ca^{2+}$)	2	1	1	3
None ($+Ca^{2+}$)	100	100	100	100
Arachidonate ($-Ca^{2+}$)	3	31	3	50
Arachidonate ($+Ca^{2+}$)	27	73	46	82
Oleate ($-Ca^{2+}$)	3	25	3	61
Oleate ($+Ca^{2+}$)	59	79	59	90
Linoleate ($-Ca^{2+}$)	4	18	3	66
Linoleate ($+Ca^{2+}$)	23	46	22	48

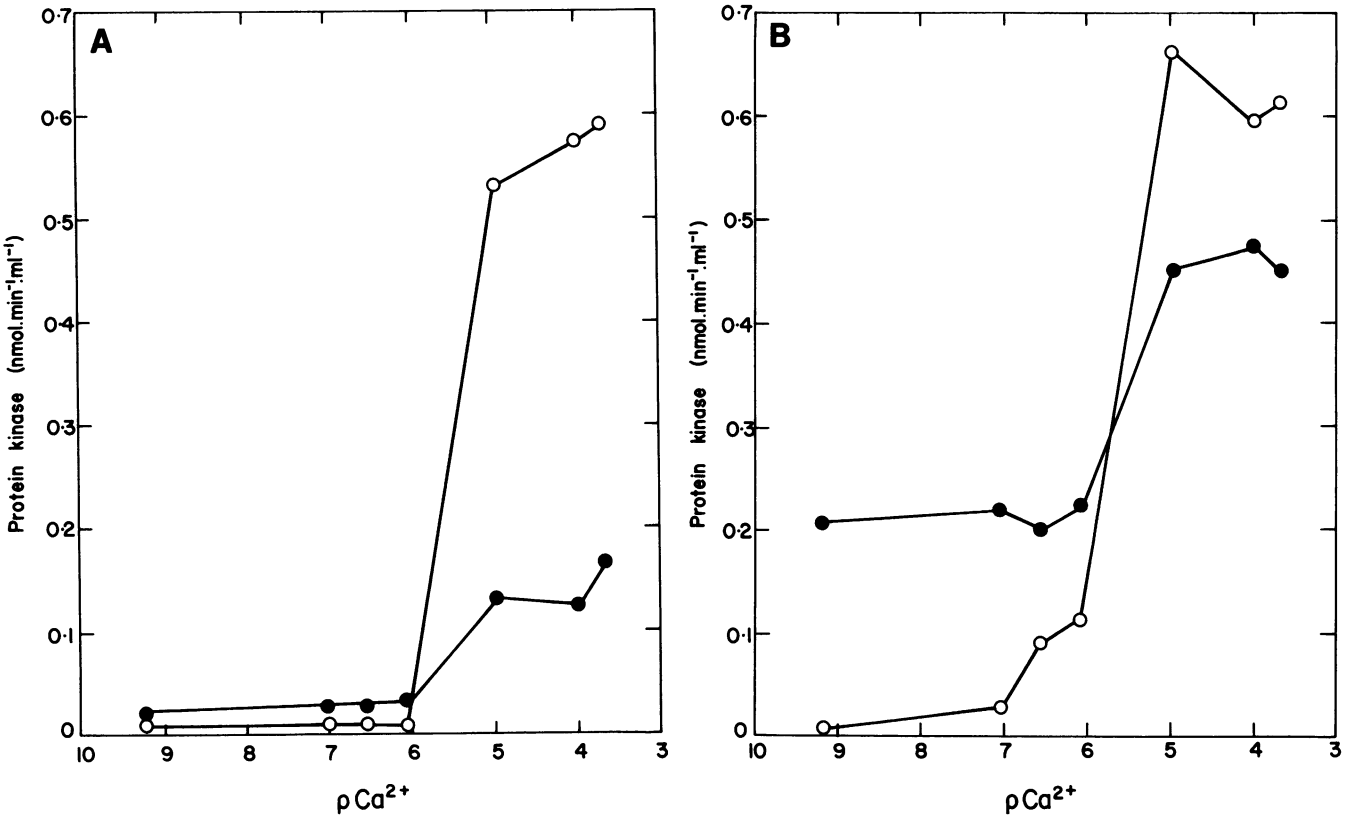

FIG. 9. Ca^{2+}-independent activation of CDPK enzymes PI and PII by arachidonate. (A) CDPK PI: (B) CDPK PII. Protein kinase (nmol min^{-1}. mL^{-1}) with 1 mg/mL histone (type III-S) as substrate was determined in the standard reaction mixture containing 0.4 mM EGTA and varying levels of added $CaCl_2$ in the presence (●——●) and absence (○——○) of 0.4 mM arachidonate. Free Ca^{2+} concentrations were calculated as described previously (14). pCa^{2+} is $-\log_{10}$ (free Ca^{2+} concentration [M]). The amount of protein kinase used in the assays was 14.7 and 15.3 pmol min^{-1} for (A) and (B), respectively.

unsaturated fatty acids in the absence of Ca^{2+} (10). However, the silver beet leaf CDPKs (16), like the soybean (5) and wheat germ (11, 14) CDPKs, are not activated by phospholipids, unlike Ca^{2+}- and phospholipid-activated protein kinase C (13).

Protein kinase C can be membrane-bound (13). Indeed, in certain conditions involving cell stimulation, protein kinase C is substantially removed from the soluble phase and relocated to membranes (13). It is not known whether such a major soluble phase to membrane-bound relocation of CDPK occurs in plant systems. The greater abundance of PI over PII in leaf membrane extracts (as compared to a greater abundance of II over I in the soluble phase) suggests a differential binding of the type I enzyme to membranes. In this connection, it should be noted that PI and enzyme I are more markedly inhibited by fatty acids in the presence of Ca^{2+} than are PII and enzyme II (Table II; Fig. 9). The differential fatty acid activation, membrane association, and substrate specificities of the types I and II silver beet leaf CDPKs could reflect differential involvement of these enzymes in plant cell responses to external stimuli mediated by free Ca^{2+} or fatty acid concentration changes. Indeed, multiple forms of protein kinase C are present in animal cells (13), and these enzymes can differ markedly in tissue distribution and in Ca^{2+}-modulated fatty acid activation properties (22).

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