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Purification and Characterization of a Ca^{2+}-Dependent Protein Kinase from the Halotolerant Green Alga *Dunaliella tertiolecta* [1]

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A Ca^{2+}-dependent protein kinase (CDPK) that has been partially purified and characterized previously [Yuasa and Muto (1992) *Arch. Biochem. Biophys.* 296: 175] was further purified to about 20,000-fold from the soluble fraction of *Dunaliella tertiolecta*. The enzyme preparation contained 60- and 52-kDa polypeptides both of which phosphorylated casein as a substrate. Both polypeptides showed a Ca^{2+}-dependent increase in mobility during SDS-PAGE and $^{45}Ca^{2+}$-binding activity after SDS-PAGE and electroblotting onto a nitrocellulose membrane, suggesting that both the 60- and 52-kDa CDPKs directly bind Ca^{2+}. The protein kinase inhibitors, K-252a and staurosporine, inhibited the CDPK competitively with respect to ATP. An antibody raised against the 60-kDa CDPK crossreacted with both the 60- and 52-kDa polypeptides. Both molecular species were autophosphorylated in the presence of Ca^{2+}, and a highly phosphorylated 80-kDa band appeared in addition to these phosphorylated bands at 60 and 52 kDa in SDS-PAGE. However, the specific activity of CDPK was not changed by prior autophosphorylation when the autophosphorylated enzyme was assayed as a mixture of these phosphorylated molecular species. Only the 60-kDa polypeptide was immunodetected in subcellular fractions of *Dunaliella* cells. The 52-kDa polypeptide increased during storage of the enzyme. These results suggest that the 52-kDa polypeptide is a proteolytic artifact produced during purification. Immunoreactive bands of 60-kDa were detected in extracts of several green algae but not in extracts of higher plants or a brown alga.

Key words: Calcium — CDPK — *Dunaliella* — Green alga — Protein kinase (EC 2.7.1.37).

Protein phosphorylation is the most important biochemical process in Ca^{2+}-mediated signal transduction in eukaryotic cells in response to external stimuli. Studies on Ca^{2+}/calmodulin-dependent protein kinase and protein kinase C have established that these second messenger-regulated protein kinases are involved in a pleiotropic control of many cellular processes by phosphorylating numerous substrates and thereby integrating related functions (Hanson and Shulman 1992, Nishizuka 1992). Recently, a new type of protein kinase, Ca^{2+}-dependent protein kinase (CDPK2) was identified (Harper et al. 1991). The CDPK requires only micromolar concentrations of Ca^{2+} for its activity. The CDPK-related protein kinases have been reported in protists (Gundersen and Nelson 1987), higher plants (Harmon et al. 1987) and green algae (Guo and Roux 1990, Yuasa and Muto 1992a). Using nucleotide probes based on the amino acid sequence derived from proteolytic fragments of a purified soybean CDPK, the first gene encoding a CDPK (CDPK-α) was cloned from soybean (Harper et al. 1991). The amino acid sequence predicted from the cDNA of CDPK-α confirmed unique structural and functional properties. The amino-terminal region of the sequence possesses considerable similarity to the catalytic domains

Abbreviations: CaMPK II, Ca^{2+}/calmodulin-dependent protein kinase II; CDPK, Ca^{2+}-dependent protein kinase; K-252a, (8*R**, 9*S**, 11*S**)-(−)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7b,11a-triazadibenzo[*a*,*g*]cyclo-octa[*c*,*d*,*e*]-trinden-1-one; PBS, 10 mM phosphate-K (pH 7.2) containing 150 mM NaCl; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

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of serine/threonine-type protein kinases and the carboxyl-terminal region, a putative regulatory domain with homology to calmodulin, including four putative Ca^{2+}-binding sites characteristic of the E–F hand family. The predicted structure of CDPK-*α* is expected to be regulated by Ca^{2+} because the biochemical properties of the purified soybean CDPK are distinct from any other known protein kinases, including Ca^{2+}-stimulated protein kinases, Ca^{2+}/calmodulin dependent-protein kinase and protein kinase C. Subsequently, cDNA and genomic clones predicted to encode the protein kinase sharing common structural characteristics of CDPK have been cloned from *Daucus carota* (Suen and Choi 1991), *Arabidopsis thaliana* (Harper et al. 1993), *Oryza sativa* (Kawasaki et al. 1993) and *Zea mays* (Estruch et al. 1994).

The green alga *Dunaliella*, which has no rigid cell wall, can grow in halophilic conditions under a wide range of salt concentration and tolerates rapid changes in environmental osmolarity. When these cells are subjected to hypo- or hyperosmotic shock, they transiently increase or decrease their cell volume, respectively. Then they rapidly recover their original volume by regulating the intracellular glycerol content. Hypoosmotic shock induces transient phosphorylation of specific proteins in *Dunaliella* cells (Ha and Thompson 1992, Yuasa and Muto 1992b). Our previous study using a protein kinase inhibitor and a Ca^{2+} ionophore suggested that the hypoosmotic shock-stimulated protein phosphorylation in *Dunaliella* cells was possibly regulated by the intracellular Ca^{2+} concentration and CDPK (Yuasa and Muto 1992b). We have partially purified CDPK from *D. tertiolecta* cells and characterized its enzymatic properties. It was shown to reversibly associated with the microsomes and preferentially phosphorylated microsomal proteins when activated by Ca^{2+} (Yuasa and Muto 1992a).

In the present study, we purified this CDPK to about 20,000-fold from the soluble fraction of *D. tertiolecta* cells and further characterized its enzymatic properties, autophosphorylation, localization and other properties.

Materials and Methods

Reagents—Dephosphorylated casein, bovine serum albumin, phosphothreonine, phosphotyrosine, phosphoserine and molecular weight standards for SDS-PAGE were purchased from Sigma. [γ-^{32}P]ATP (111 TBq mmol^{-1}) and $^{45}CaCl_2$ (370 GBq (mg calcium)$^{-1}$) were obtained from NEN. Phenyl-Sepharose CL4B, prestained molecular weight markers (rainbow markers) and dithiothreitol were purchased from Pharmacia, Amersham and Boehringer Mannheim, respectively. Polyvinylidene difluoride membrane, nitrocellulose membrane, DEAE-Toyopearl 650M, ATP, horseradish peroxidase-conjugated recombinant protein G and Coomassie Brilliant Blue R 250 were products of Millipore, Schleicher & Schuell, TOSO, Yamasashoyu, Zymed and Fluka, respectively. Cellulose thin layer plates, K-252a and staurosporine were purchased from Funakoshi. All other reagents used were of highest quality.

Algal culture—D. tertiolecta was grown as previously described (Yuasa and Muto 1992a). Growth conditions of *D. salina* and *Chlamydomonas reinhardtii* were essentially the same as those of *D. tertiolecta* except that each medium contained 2 M and 0 M NaCl, respectively.

Preparation and assay of CDPK—The purification steps up to the DEAE-Toyopearl 650M column were essentially the same as previously described (Yuasa and Muto 1992a). Briefly, the algal cell extract (from 200,000 × *g* supernatant of cells) was treated with ammonium sulfate at 40–65% saturation. The resultant precipitate was dissolved and chromatographed on a DEAE-Toyopearl 650M column (3.4 × 25 cm). The pool of fractions containing CDPK activity was directly applied to a Phenyl-Sepharose CL-4B column (1.3 × 10 cm) equilibrated with 25 mM HEPES-KOH (pH 7.6) containing 0.5 mM EGTA, 0.5 mM EDTA, 1 mM $PhMeSO_2F$, 1 mM dithiothreitol and 150 mM KCl (buffer A) at a flow rate of 0.2 ml min^{-1}. The column was successively washed with 50 ml of the same buffer, 50 ml of 25 mM HEPES-KOH (pH 7.6) containing 0.5 mM $CaCl_2$ (buffer B) and 300 ml of 20 mM Tris-HCl (pH 8.5) containing 0.1 mM $CaCl_2$ and 40% ethyleneglycol (buffer C) at a flow rate of 1 ml min^{-1}. The CDPK was eluted with 20 mM Tris-HCl (pH 8.5) containing 1 mM EGTA and 40% ethyleneglycol (buffer D) at a flow rate of 0.2 ml min^{-1}. The CDPK was highly purified at this step. Thus the hydroxylapatite step in the previous method (Yuasa and Muto 1992a) was omitted. The enzyme preparation was stored at −70°C.

The assay of CDPK activity was carried out at 30°C as previously described with casein as substrate (Yuasa and Muto 1992a).

Preparation of crude subcellular fractions from Dunaliella cells—Cell cultures (500 ml, 0.2% packed cell volume) were harvested by centrifugation at 900 × *g* for 4 min at 15°C. After washing with 50 mM Tris-HCl (pH 7.5) containing 2 mM $MgCl_2$ and 0.5 M NaCl and then with 10 mM MES-Tris (pH 7.0) containing 0.5 mM $MgCl_2$ and 0.8 M glycerol, the cells (1 ml packed cell volume) were resuspended in 10 ml of cold 10 mM MES-Tris (pH 7.0) containing 5 mM EDTA, 5 mM EGTA, 2.5 mM dithiothreitol, 2.5 mM $PhMeSO_2F$, 1 mM benzamidine and 5 mM *n*-aminocaproic acid. To osmolyse the cells, the suspension was gently shaken for 60 min at 4°C. The crude cell lysate was centrifuged at 10,000 × *g* for 10 min at 4°C. The resultant supernatant fraction was centrifuged at 200,000 × *g* for 15 min at 4°C. All buffers used thereafter contained 1 mM dithiothreitol, 1 mM $PhMeSO_2F$, 1 mM benzamidine and 5 mM *n*-aminocaproic acid. The 10,000 × *g* and 200,000 × *g* pellets were separately resuspended in 10 mM MES-Tris

(pH 7.0) containing 0.5 mM EDTA, 0.5 mM EGTA and 1 M glycerol, and then homogenized with a Dounce homogenizer. The 200,000 × *g* pellet was resuspended and centrifuged as above to obtain washed microsomes. The volume of all fractions was adjusted to 10 ml. The distribution of CDPK was analyzed by immunoblotting with anti-CDPK antibody.

SDS-PAGE and autoradiography—SDS-PAGE was done according to the method of Laemmli (1970) using 10% acrylamide and 0.27% bisacrylamide. Phosphorylated proteins were precipitated by 10% (w/v) trichloroacetic acid and the precipitates were rinsed with cold 250 mM sucrose. After neutralization with 1 M Tris, the precipitates were dissolved in SDS-PAGE sample buffer. After electrophoresis gels were stained and dried on filter paper. Autoradiography of the dried gels were performed with X-ray film using an intensifying screen (Lightning Plus, Dupont).

^{45}Ca-binding assay—$^{45}Ca^{2+}$-binding to CDPK polypeptides on a nitrocellulose membrane was performed according to the method of Maruyama et al. (1984), with some modifications. CDPK polypeptides were electroblotted onto nitrocellulose membrane (pore size, 0.45 μm) as in western blotting. After electroblotting, the membrane was soaked five times within 1 h with 10 mM imidazole-HCl (pH 6.8) containing 60 mM KCl, 5 mM $MgCl_2$ and 0.05% (v/v) Tween 20 to wash out the electrode buffer. Then the membrane was incubated in 15 mM Na-acetate (pH 5.2) containing 60 mM KCl, 5 mM $MgCl_2$ and 0.05% (v/v) Tween 20 for 5 min in order to release Ca^{2+} bound to the Ca^{2+}-binding proteins. ^{45}Ca (37 kBq ml^{-1}) was added and the pH of the incubation mixture was adjusted to 7.5 by the addition of 1 M Tris base. After incubating for 10 min at room temperature, the membrane was rinsed with distilled water for 5 min, dried at room temperature for 1 h, and then autoradiographed.

Preparation of anti-CDPK polyclonal antibody—CDPK (500 μg protein) was electrophoresed in an SDS-polyacrylamide gel. The gel was stained with Coomassie blue and destained in 10% (v/v) acetic acid four times for 20 min each. The protein band with a molecular mass of 60 kDa was excised and then washed with 50 mM $NaHCO_3$ for 5 min. After equilibrating the gel piece with 62.5 mM Tris-HCl (pH 6.7), 0.1% (w/v) SDS and 5 mM dithiothreitol for 1.5 h, the protein was electroeluted at 100 V for 4 h by an electroelutor (Model 1750, ISCO) and vacuum-dried. The protein was solubilized in PBS and then injected subcutaneously into a rabbit.

Western blotting—Proteins in an SDS-PAGE gel were electroblotted onto a polyvinylidene difluoride membrane as described previously (Shimogawara and Muto 1989). After blocking with PBS containing 5% (w/v) skim milk and 0.05% (v/v) Tween 20 (blocking buffer) for 1 h, the membrane was treated for 2 h with anti-CDPK rabbit antiserum diluted 1/2,000 (v/v) with the blocking buffer. The membrane was washed three times with the blocking buffer and incubated with 1/2,000-diluted horse radish peroxidase-conjugated recombinant protein G. After washing three times with the blocking buffer and once with PBS containing 0.05% (v/v) Tween 20, the immunodecorated protein bands were visualized by incubating the membrane in 100 mM Na-citrate (pH 4.2) containing 1 mM diaminobenzidine and 0.03% (v) H_2O_2.

In-gel protein kinase assay in an SDS-PAGE gel containing casein—In-gel protein kinase activity in a polyacrylamide gel was detected according to the method of Guo and Roux (1990). CDPK was electrophoresed in a 10% polyacrylamide gel containing 1 mg ml^{-1} casein (casein-gel). To remove SDS and renature CDPK, the gel was washed four times for 30 min each with 25 mM Tris-HCl (pH 7.5) containing 1 mM dithiothreitol at room temperature and then gently shaken at 4°C for 15 h in the same buffer. After equilibrating the gel with 25 mM HEPES-KOH (pH 7.6) containing 5 mM $MgCl_2$, 150 μM $CaCl_2$ and 1 mM dithiothreitol, 50 μM [γ-^{32}P]ATP (370 kBq ml^{-1}) was added and incubated at room temperature for 5 h with gentle shaking. The reaction was terminated by transferring the gel to 5% (w/v) trichloroacetic acid containing 1% (w/v) $Na_4P_2O_7 \cdot 10H_2O$. The gel was washed with the same solution 10 times for 20 min each and then washed in a fixing solution composed of acetic acid, methanol and distilled water (20 : 7 : 73, by volume). The gel was dried on filter paper and autoradiographed as described above.

Phosphoamino acid analysis—Autophosphorylated CDPK was precipitated with 10% (w/v) trichloroacetic acid, rinsed with acetone twice, and then partially hydrolyzed with 6 M HCl at 105°C for 2 h. Phosphoamino acids were identified by two-dimensional thin layer electrophoresis as previously described (Yuasa and Muto 1992a).

Determination of protein concentration—Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as standard.

Results

Purification of Dunaliella CDPK—Hydrophobic chromatography on a Phenyl-Sepharose CL-4B column was very efficient for purifying this CDPK (Table 1). Almost all of CDPK activity was retained on the column in the presence of 0.1 mM $CaCl_2$ in buffer C, and then eluted by buffer D containing 1 mM EGTA as a double peak (Fig. 1). Starting with 150 liters of culture (packed cell volume, 0.12 to 0.15%), 0.46 mg purified CDPK with a specific activity of 889 nmol min^{-1} (mg protein)$^{-1}$ was obtained. The enzymatic properties of the purified enzyme, including its Ca^{2+}-dependency, pH dependency, substrate specificity and apparent K_ms for ATP and casein, were essentially identical to those of the partially purified CDPK

Table 1 Purification of CDPK from *Dunaliella tertiolecta* cells

Purification step	Protein (mg)	Total activity (nmol min^{-1})	Specific activity (nmol min^{-1} (mg protein)$^{-1}$)	Purification (x-fold)
200,000 × *g* supernatant	4,400	190	0.043	1
Ammonium sulfate 40–65% saturation	2,130	298	0.140	3
DEAE-Toyopearl 650M	141	465	3.30	77
Phenyl-Sepharose CL4B	0.46	409	889	20,700

previously described (Yuasa and Muto 1992a). The purified enzyme peparation showed a major 60-kDa polypeptide and a minor 52-kDa polypeptide following SDS-PAGE (Fig. 2; lane 4). Both the 60- and 52-kDa polypeptides showed a Ca^{2+}-dependent increase in mobility during SDS-PAGE, resulting in a 57-kDa major band and a minor doublet around 48 kDa (Fig. 2; lane 5). This Ca^{2+}-dependent mobility shift of the CDPK polypeptides in SDS-PAGE suggests that they contain a Ca^{2+}-binding domain like other CDPKs in higher plants (Roberts and Harmon 1992).

Ca^{2+}-binding activity of CDPK—To examine directly Ca^{2+} binding to CDPK, the enzyme was transferred onto a nitrocellulose membrane after SDS-PAGE, and incubated with $^{45}Ca^{2+}$. An autoradiogram of the membrane showed a major 60-kDa and a 52-kDa minor radioactive band (Fig. 3, lane 1), indicating that the CDPK directly bound Ca^{2+}.

In-gel protein kinase activity in a casein-gel—Two polypeptides with molecular masses of 60 and 52 kDa were copurified but it was unclear whether both have Ca^{2+}-dependent protein kinase activity. Figure 4 shows that protein kinase activity was detectable in the samples from all purification steps by an in-gel protein kinase assay in a casein-gel. The Phenyl-Sepharose fraction (lane 4) showed two activity bands at 60 and 52 kDa, indicating that both polypeptides detected in the Coomassie-stained gel (Fig. 2) have protein kinase activity. The DEAE-Toyopearl fraction (lane 3) showed a 40-kDa band in addition to the 60- and 52-kDa bands. The ammonium sulfate precipitate (lane 2) and the 200,000 × *g* supernatant fraction (lane 1) showed a pattern similar to that of the DEAE-Toyopearl fraction except that the 52-kDa band appeared as a doublet. The 40-kDa band was detected in the presence or

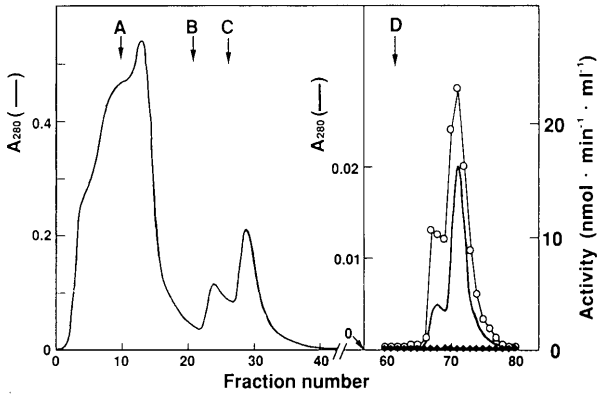

Fig. 1 Phenyl-Sepharose CL4B hydrophobic chromatography of CDPK. Elution was carried out as described in Materials and Methods. Protein profile was monitored at A_{280} (thick line). Fraction size was 9 ml $tube^{-1}$. The arrows refer to the start of the corresponding buffers (see Materials and Methods). The break in the *x* axis between 40th and 60th fraction is omitted for clarity. The protein kinase activity, in the presence (open circles) and absence (closed square) of Ca^{2+}. The protein kinase activity of the pass-through fraction and washings with buffer A, B and C are omitted for clarity.

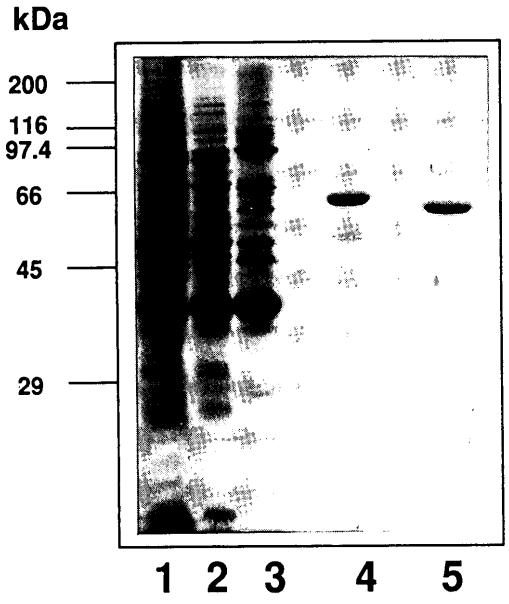

Fig. 2 Purification of CDPK as followed by SDS-PAGE. Preparations of each purification steps of *Dunaliella* CDPK were subjected to SDS-PAGE (10% acrylamide) and stained with Coomassie Brilliant Blue R250. 200,000 × *g* supernatant (30 μg protein), lane 1; precipitate at 40–65% saturation of ammonium sulfate (25 μg), lane 2; DEAE-Toyopearl pool (20 μg), lane 3; Phenyl-Sepharose pool (2 μg), 2 mM EGTA (lane 4) or 2 mM $CaCl_2$ (lane 5) was included in the sample buffer. The relative molecular masses of molecular standards are indicated on the left side of the gel.

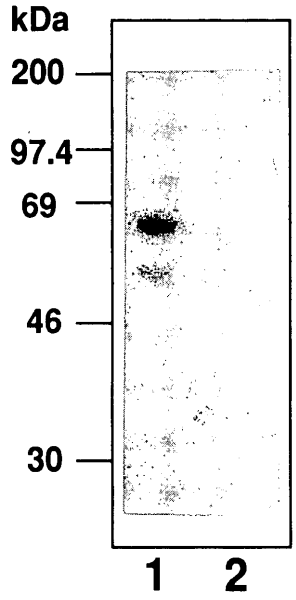

Fig. 3 $^{45}Ca^{2+}$ binding to purified CDPK. Samples were subjected to SDS-PAGE, electroblotted onto nitrocellulose membrane, incubated with $^{45}Ca^{2+}$, and rinsed with distilled water for 5 min. Lane 1, 0.5 μg purified CDPK; lane 2, 5 μg prestained markers. Note that there is no nonspecific binding of $^{45}Ca^{2+}$ to the marker polypeptides.

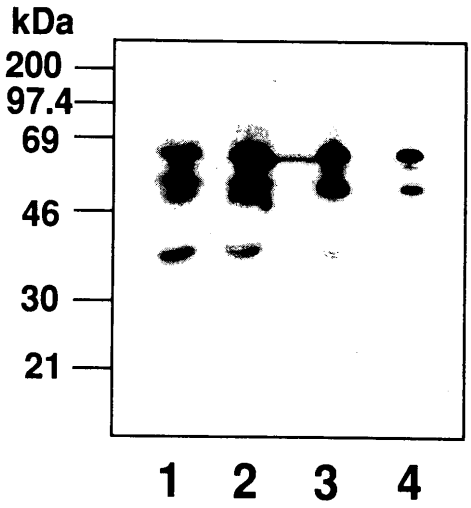

Fig. 4 In-gel detection of protein kinase activity in casein-gel. Preparations of each purification steps of *Dunaliella* CDPK were subjected to SDS-PAGE of casein-gel (10% acrylamide, 1 mg (ml casein)$^{-1}$) and the enzyme activity was assayed as described in Materials and Methods. 200,000 × *g* supernatant (60 μg protein), lane 1; precipitate at 40–65% saturation of ammonium sulfate (40 μg), lane 2; DEAE-Toyopearl pool (2 μg), lane 3; Phenyl-Sepharose pool (30 ng), lane 4. Indicated on the left side of the autoradiogram are the relative molecular masses of prestained protein markers (rainbow markers).

absence of Ca^{2+} in the in-gel reaction mixture, whereas the other phosphorylated bands were detected only in the presence of Ca^{2+} (data not shown). These results indicate that the 40-kDa band is a Ca^{2+}-independent protein kinase and the other bands are CDPKs. A Ca^{2+}-dependent mobility shift was also observed in the in-gel protein kinase assay in casein-gel with the purified enzyme (Fig. 5). However, the minor band was not a doublet, suggesting that one of the doublet bands detected by protein staining (Fig. 2) has no protein kinase activity.

Anti-60-kDa CDPK antibody—A rabbit polyclonal antibody was raised against the denatured 60-kDa CDPK polypeptide (Fig. 2, lane 4). This antibody preparation inhibited CDPK activity when they were preincubated together before the enzyme assay. The extent inhibition was 50% at a 1/2,000 dilution of the antibody. Rabbit preimmune serum showed no effect on CDPK activity at a concentration lower than a 1/30 dilution (data not shown). Anti-60-kDa CDPK antibody cross-reacted with both the 60- and 52-kDa bands in the purified CDPK preparation (see Fig. 6C, 2 min), but only a 60-kDa immunoreactive band was detected in the *Dunaliella* cell extract (see Fig. 10, lane 1). The single immunoreactive band in the *Dunaliella* cell extract showed a Ca^{2+}-dependent mobility shift as observed with the purified CDPK (data not shown).

Effects of K-252a and staurosporine on CDPK activity—We have reported that the partially purified CDPK from *Dunaliella* is inhibited by K-252a, an indolocarbazole derivative, but not by H-9, an isoquinorine sulfonamide derivative (Yuasa and Muto 1992a). Inhibition kinetics of the purified CDPK by K-252a and staurosporine were examined. K-252a and staurosporine inhibited CDPK competitively

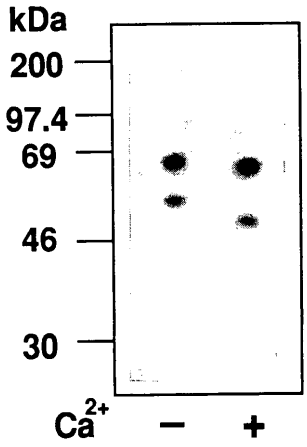

Fig. 5 Ca^{2+}-dependent mobility shift of purified CDPK in casein-gel. In-gel protein kinase assay was performed as described in Materials and Methods. The sample buffer for SDS-PAGE contained 2 mM EGTA (Ca^{2+}: −) or 2 mM $CaCl_2$ (Ca^{2+}: +).

with respect to ATP (data not shown). The K_i values for K-252a and staurosporine were estimated to be 2.5 μM and 75 nM, respectively.

Autophosphorylation of CDPK—It was reported that protein kinase C and CaMPK II are autophosphorylated and that this modification results in their properties (Huang et al. 1986, Schulman and Lou 1989). Autophosphorylation of a purified soybean CDPK preparation has been detected in an in-gel protein kinase assay following SDS-PAGE (Harmon et al. 1987), but it was unclear whether autophosphorylation of CDPK affected its activity or other properties. We were able to detect autophosphorylation of the partially purified *Dunaliella* CDPK (Yuasa and Muto 1992a). Hence, autophosphorylation was re-examined with the purified enzyme. When the purified *Dunaliella* CDPK was incubated with Mg^{2+}-[γ-^{32}P]ATP and Ca^{2+} for up to 90 min, ^{32}P was incorporated into one major and two minor bands with molecular masses of 80 kDa, 60 and 52 kDa, respectively (Fig. 6B). Coomassie staining showed that the amount of the 60-kDa polypeptide slightly decreased and a new band appeared at 80 kDa after autophosphorylation for 30 min, while the 52-kDa polypeptide was not altered (Fig. 6A). The new band at 80 kDa was detected by western blotting with anti-CDPK antibody (Fig. 6C), indicating that this polypeptide is immunologically related to the 60- and 52-kDa polypeptides. When CDPK was incubated in the same conditions except for the absence of Ca^{2+}, no ^{32}P incorporation into the CDPK polypeptides was detected after 60-min (data not shown). These results suggest that the 80-kDa polypeptide was derived from the 60-kDa polypeptide by autophosphorylation and that the autophosphorylation of CDPK required Ca^{2+}. The relative proportion of ^{32}P incorporated into these bands was remarkably different from the intensities following Coomassie-staining or western blot (Fig. 6). The phosphoprotein bands from the 90-min sample were cut out and their radioactivities were measured. Seventy-two percent of the total ^{32}P incorporated was distributed in the 80-kDa polypeptide, as compared to 21 and 7% in the 60- and 52-kDa polypeptides, respectively. Obviously, the 80-kDa polypeptide has the highest specific radioactivity and the 60-kDa band has the lowest. To examine whether the 80-kDa polypeptide has protein kinase activity, an in-gel protein kinase assay of autophosphorylated CDPK was carried out in a casein-gel. Three bands appeared at 80, 60 and 52 kDa, indicating that the 80-kDa band has protein kinase activity as well as the 60- and 52-kDa bands (Fig. 7).

Next, the effect of prior autophosphorylation on the activity of CDPK was examined. After incubating CDPK with Mg^{2+}-ATP in the presence or absence of Ca^{2+}, the protein kinase activity was measured. Essentially no change in CDPK activity was observed (Fig. 8, $+Ca^{2+}$). Ca^{2+}-independent activity, which has been reported to appear in CaMPK II after autophosphorylation (Hanson and Schulman 1992), was not detected in CDPK (Fig. 8, $-Ca^{2+}$).

The phosphoamino acid(s) in autophosphorylated CDPK was analyzed by two-dimensional cellulose thin layer electrophoresis (Fig. 9). Phosphoserine and phosphothreonine were detected and the former was dominant.

Subcellular localization of CDPK in Dunaliella cells—Crude subcellular fractions were prepared from osmotical-

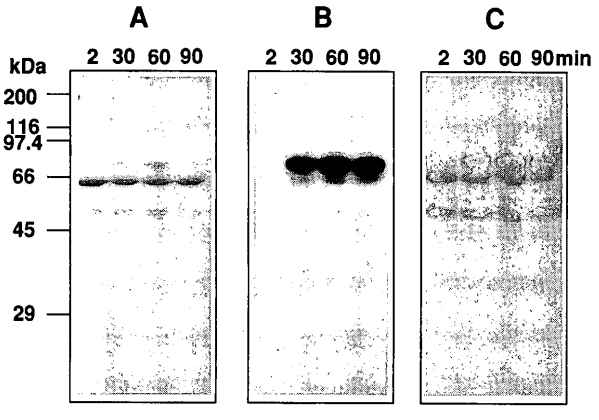

Fig. 6 Autophosphorylation of CDPK. CDPK was incubated with [γ-^{32}P]ATP without external substrate protein in the presence of 150 μM $CaCl_2$. Aliquots of the reaction mixtures (containing 0.8 μg of the enzyme) were withdrawn at the indicated time points and the reaction was stopped by adding 10% (final concn.) trichloroacetic acid. Precipitated polypeptides were subjected to SDS-PAGE followed by autoradiography and western blotting with anti-CDPK antibody. Coomassie Brilliant blue staining, panel A; autoradiography, panel B; western blot, panel C.

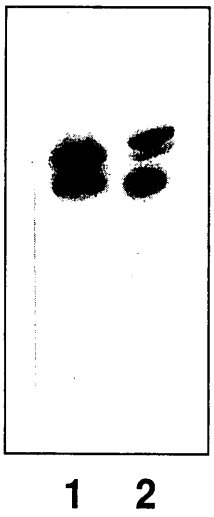

Fig. 7 In-gel detection of protein kinase activity of autophosphorylated CDPK in casein-gel. Autophosphorylation assay was performed for 30 min as in Fig. 6, and stopped by adding 1 mM EGTA and transferring on ice. In-gel protein kinase assay was performed as in Materials and Methods. Control (50 ng), lane 1; autophosphorylated CDPK (50 ng), lane 2.

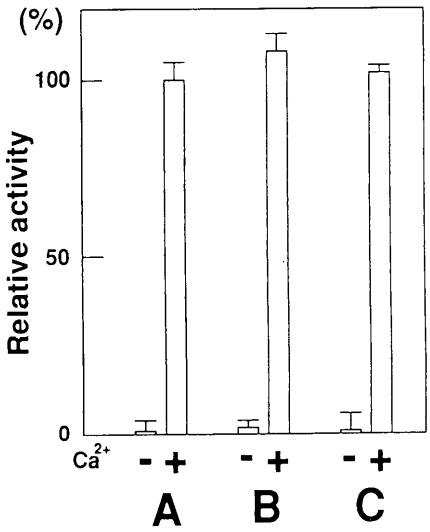

Fig. 8 Effect of autophosphorylation on CDPK activity. Autophosphorylation reaction was performed in the absence (1 mM EGTA; B) or the presence (150 μM $CaCl_2$; C) of Ca^{2+}. The reaction was stopped as in Fig. 8. Ca^{2+}-dependent protein kinase activity in control (without autophosphorylation reaction; A) was 430 pmol min^{-1} $reaction^{-1}$. Values are the averages of duplicates.

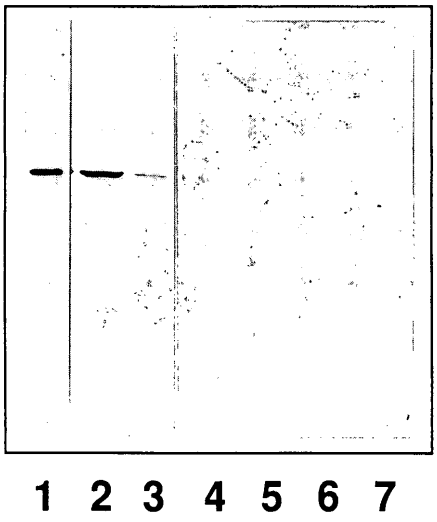

Fig. 10 Western blot analysis of cell extracts of algae and higher plants by anti-CDPK antibody. SDS-PAGE and western blotting were performed as described in Materials and Methods. Lane 1, *D. tertiolecta* (15 μg protein); lane 2, *D. salina* (15 μg); *Chlamydomonas reinhardtii* (15 μg); lane 4, *Oryza sativa* (60 μg); lane 5, *Nicotiana tabacum* BY-2 (50 μg); lane 6, *Arabidopsis thaliana* (30 μg); lane 7, *Undaria pinnetifida* (15 μg).

ly lysed *Dunaliella* cells by differential centrifugation and analyzed subsequently by western blotting. Only the 60-kDa band was detected in all subcellular fractions (data not shown). This indicates that *Dunaliella* CDPK consists of only the 60-kDa polypeptide and that the 52-kDa polypeptide in the (partially) purified CDPK preparations artificially produced from the 60-kDa. Densitometric analysis of the immunoblots indicated that approximately 64% of the total cellular 60-kDa CDPK is found in the 10,000 × *g* supernatant fraction. After re-centrifugation of this fraction at 200,000 × *g*, 55% of the total 60-kDa CDPK was found in the 200,000 × *g* supernatant fluid. The CDPK contained in the 200,000 × *g* pellet was remarkably decreased after washing. These results suggest that the majority of the 60-kDa CDPK was localized in the soluble fraction of *Dunaliella* cells. Thirteen percent of the total CDPK remained in the washed microsomes. The membrane-bound CDPK was solubilized only after treatment of the microsomes with urea or detergent (data not shown).

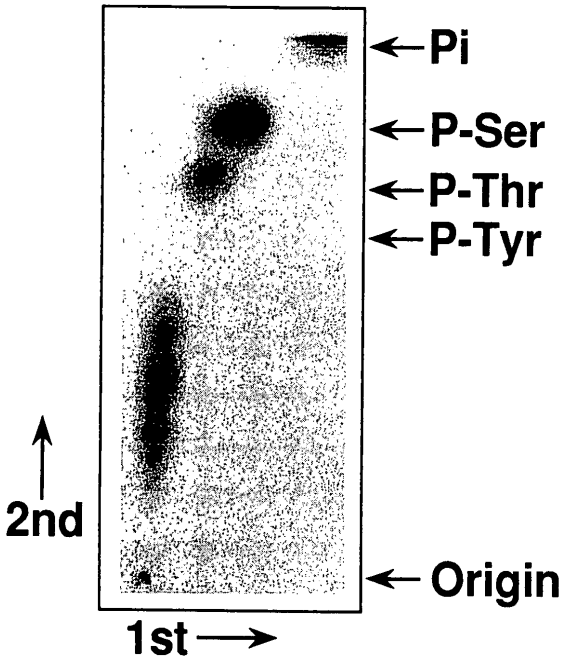

Fig. 9 Identification of phosphoamino acids in autophosphorylated CDPK. Autophosphorylated CDPK was hydrolyzed with 6 M HCl at 105°C for 2 h and subjected to two-dimensional thin layer cellulose electrophoresis as described in Materials and Methods.

Distribution of 60-kDa CDPK in algae and higher plants—The anti-60-kDa CDPK antibody also cross-reacted with a 60-kDa polypeptide in cell extracts of *D. salina* and *Chlamydomonas reinhardtii* (Fig. 10). No immunoreactive band was detected in cell extracts of three higher plants i.e., suspension cultures of *Oryza sativa*, *Nicotiana tabacum* BY-2 and *Arabidopsis thaliana*, and in zoospores of the brown alga, *Undaria pinnetifida*.

Discussion

A CDPK has been highly purified from *D. tertiolecta*

cells. This enzyme is the fifth member of the CDPK family so far characterized in detail. The CDPK family includes CaPK-1 (Son et al. 1993) and CaPK-2 (Gundersen and Nelson 1987) purified from *Paramecium tetraurelia* cells, soybean CDPK (Putnam-Evans et al. 1990) and groundnut CDPK (DasGupta 1994). Several properties of *Dunaliella* CDPK described in the present report are similar to those of soybean CDPK and *Paramecium* CaPK-2, i.e., (i) the relative molecular mass of 50 to 60-kDa, (ii) the Ca^{2+}-dependent mobility shift in SDS-PAGE, (iii) the activation by micromolar concentrations of Ca^{2+}, (iv) the direct binding of Ca^{2+}, and (v) a Ca^{2+}-dependent autophosphorylation. It was reported that *Paramecium* CaPK-1 was autophosphorylated in the presence or absence of Ca^{2+} and did not show a Ca^{2+}-dependent mobility shift (Son et al. 1993). *Dunaliella* CDPK (Yuasa and Muto 1992a) and *Paramecium* CaPK-2 (Gundersen and Nelson 1987) preferentially phosphorylated casein, whereas soybean CDPK did not (Putnam-Evans et al. 1990). *Dunaliella* CDPK (Yuasa and Muto 1992a) and *Paramecium* CaPK-2 (Gundersen and Nelson 1987) are inhibited by sulfhydryl reagents and are unstable in the absence of dithiothreitol, suggesting the requirement of -SH groups for their activities, while soybean CDPK does not require such protection of -SH groups for its activity (Putnam-Evans et al. 1990). Thus, *Dunaliella* CDPK resembles *Paramecium* CaPK-2 rather than soybean CDPK.

The CDPK-related protein kinases in higher plants have been localized in the soluble fraction of soybean (Putnam-Evans et al. 1990), the plasma membrane of oat (Schaller et al. 1992), the nuclei of pea (Li et al. 1991) and other species (see review by Roberts and Harmon (1992)). However, none of them has been purified to homogeneity except the soluble CDPK of soybean and groundnut. The membrane-bound CDPK of oat was immunologically related to the soluble CDPK of soybean, but it required both Ca^{2+} and phospholipid for activation and its relative molecular mass was larger than that of the soluble CDPK (Schaller et al. 1992).

We have reported that *Dunaliella* CDPK reversibly associates with the microsomes in a Ca^{2+}-dependent manner and preferred the microsomal proteins to the soluble proteins as substrate (Yuasa and Muto 1992a). In the present study, it was revealed that a high concentration of ethylene glycol was required for elution of *Dunaliella* CDPK from a Phenyl-Sepharose column (Fig. 1), suggesting that *Dunaliella* CDPK has a high hydrophobicity and that this property may be responsible for its association to the microsomes.

The purified *Dunaliella* CDPK contained both 60- and the 52-kDa polypeptides, but only the 60-kDa CDPK was detected prominently in cell extracts by the western blotting (Fig. 10). The content of the 52-kDa polypeptide increased and that of the 60-kDa polypeptide decreased after storing the ammonium sulfate precipitate at −20°C for several months (data not shown). These results indicate that the 52-kDa polypeptide is a degradation product derived from the 60-kDa CDPK.

A CDPK-related protein kinase has been partially purified from *D. salina* and characterized (Guo and Roux 1990). The relative molecular mass of this algal CDPK was estimated to be about 40 kDa by in-gel protein kinase assays in an SDS-polyacrylamide gel containing histone H1. A 60-kDa band that immunoreacted with anti-*Dunaliella* CDPK antibody was detected in *D. tertiolecta, D. salina* and *Chlamydomonas reinhardtii* extracts (Fig. 10). In-gel protein kinase assays with DEAE-Toyopearl fraction prepared from *D. tertiolecta* cells showed the 60- and the 52-kDa, which appeared in a Ca^{2+}-dependent manner, and the 40-kDa band, which appeared in a Ca^{2+}-independent manner (Fig. 4 and data not shown). It was reported that the 40-kDa *D. salina* CDPK did not show a Ca^{2+}-dependent mobility shift in SDS-PAGE and that casein was a poor substrate for the enzyme (Guo and Roux 1990). These facts indicate that the 40-kDa protein kinases in *D. salina* and *D. tertiolecta* are different enzymes.

K-252a and staurosporine inhibited *Dunaliella* CDPK competitively with respect to ATP. It was reported that these indolocarbazole derivatives also inhibited several types of protein kinases with a broad range, including serine/threonine- and tyrosine-type protein kinases and their inhibition was competitively with respect to ATP in most cases (Kase et al. 1987, Herbert et al. 1990).

We have previously reported that K-252a inhibits the recovery in cell volume of *D. tertiolecta* from hypoosmotic shock and that hypoosmotic shock transiently increases phosphorylation of specific polypeptides with molecular masses of 28 and 30 to 32 kDa (Yuasa and Muto 1992b). Furthermore, the phosphorylation of these polypeptides was effectively inhibited by K-252a under hypoosmotic conditions and stimulated by the addition of A23187 under isoosmotic condition. These results suggest that K-252a-sensitive protein kinase(s) is involved in osmoregulation of *Dunaliella* cells under hypoosmotic conditions and the hypoosmotic shock-induced protein phosphorylation are regulated by the intracellular Ca^{2+} concentration. Because *Dunaliella* CDPK, which is effectively inhibited by K-252a, remarkably phosphorylated the 28-kDa protein in the microsomal fraction (Yuasa and Muto 1992b), it may be a candidate protein kinase involved in the hypoosmotic shock-induced protein phosphorylation.

In vitro autophosphorylation of CDPK produced a highly phosphorylated 80-kDa polypeptide in addition to the minor labeling of the 60- and the 52-kDa polypeptides (Fig. 6B), but did not cause a change in enzyme activity when a mixture of the polypeptides was assayed (Fig. 8). It is well known that Ca^{2+}-dependent and -independent activities of CaMPK II are regulated by autophosphorylation in

a complex manner (Hanson and Schulman 1992). When Ca^{2+}/calmodulin-complex binds to CaMPKII, Ca^{2+}-independent activity is induced within 1 min by autophosphorylation of the enzyme and autophosphorylation is a prerequisite for its full activation and phosphorylation of substrate (Kato and Fujisawa 1991). Autophosphorylation of *Dunaliella* CDPK proceeded slower than that of CaMPK II in that it was not detectable within 2 min (Fig. 6B). This may be the reason why autophosphorylation was not detected with the partially purified CDPK in our previous study (Yuasa and Muto 1992a), in addition to the fact that the previous CDPK activity was much lower than that in the present study. Autophosphorylation has been reported with CDPKs purified from soybean (Harmon et al. 1987), groundnut (DasGupta 1994) and *Paramecium* (Son et al. 1993). These reports indicated that autophosphorylation had either no effect on properties or a slight decrease in their mobility in SDS-PAGE (1–2 kDa). *Paramecium* CaPK-1 is autophosphorylated in the absence of Ca^{2+}, while plant CDPKs of soybean, groundnut and *Dunaliella* require Ca^{2+} for autophosphorylation. Although *Dunaliella* CDPK requires Ca^{2+} for autophosphorylation but *Paramecium* CaPK-1 does not, both enzymes require Ca^{2+} for phosphorylation of exogenous substrate. Ca^{2+}-independent activity was not induced in either enzyme by autophosphorylation in contrast with CaMPKII. The results in Figs. 6 and 7 may suggest that the 60-kDa polypeptide of purified *Dunaliella* CDPK composed of at least two isoforms, a minor isoform which was remarkably autophosphorylated and decreased in mobility in SDS-PAGE and another major isoform which was only slightly autophosphorylated and scarcely altered in mobility. The specific activity of these respective isoforms are not known since the activity was assayed as a mixture of them. We have already demonstrated that *Dunaliella* CDPK reversibly associates to the microsomes in vitro in a Ca^{2+}-dependent manner much like the "translocation of protein kinase C induced by activators" (Yuasa and Muto 1992a). Since the translocation and down-regulation of protein kinase C appears to be regulated by autophosphorylation in a complex manner accompanying by a mobility shift in SDS-PAGE (Borner et al. 1989), we speculate that the intracellular behavior of *Dunaliella* CDPK is possibly regulated by autophosphorylation. It is important to clarify whether autophosphorylation of CDPK occurs in vivo and causes any changes in enzymatic properties such as substrate specificity and intracellular localization, as well as specific activity.

The complete amino acid sequence of *Dunaliella* CDPK is necessary for its structural comparison with other CDPKs. We have isolated cDNA clones of *Dunaliella* CDPK by immunoscreening with the anti-*Dunaliella* CDPK antibody. Sequence analysis of these clones and characterization of the products of the cDNA clones expressed in *E. coli* cells are in progress.

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