Casein Kinase II-Type Protein Kinase from Pea Cytoplasm and Its Inactivation by Alkaline Phosphatase in Vitro[1]

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A casein kinase II-type protein kinase has been purified from the cytosolic fraction of etiolated pea (*Pisum sativum* L.) plumules to about 90% purity as judged from Coomassie blue stained sodium dodecyl sulfate-polyacrylamide gels. This kinase has a tetrameric $\alpha\alpha'\beta_2$ structure with a native molecular mass of 150 kD, and subunit molecular masses of 41 and 40 kD for the two catalytic subunits (α and α') and 35 kD for the putative regulatory subunit (β). Casein and phosvitin can be used as artificial substrates for this kinase. Both serine and threonine residues were phosphorylated when mixed casein, β-casein, or phosvitin were used as the substrate, whereas only serine was phosphorylated if α-casein or histone III-S was the substrate. The kinase activity was stimulated 130% by 0.5 mM spermine (the concentration required for 50% of maximal enzyme activity [A_{50}] = 0.1 mM) and 80% by 2.5 mM spermidine (A_{50} = 0.4 mM), whereas putrescine and cadaverine had no effect. The kinase was very sensitive to inhibition by heparin (concentration for 50% inhibition [I_{50}] = 0.025 μg/mL). In contrast to most other casein kinase II-type protein kinases, this preparation was inhibited by K^+ and Na^+, with I_{50} values of 75 and 65 mM, respectively. Pretreatment of the purified kinase preparation in vitro with alkaline phosphatase caused a 5-fold decrease in its activity. Additionally, this kinase also lost its activity when its β subunit was autophosphorylated in the absence of substrate. These results suggest that the activity of this casein kinase II protein kinase may be regulated by the phosphorylation state of two different sites in its multimeric structure.

CK II is a multifunctional, cyclic nucleotide- and calcium-independent, Ser/Thr-specific protein kinase (Pinna, 1990; Tuazon and Traugh, 1991). Current evidence shows that CK II kinases play important roles in a variety of processes, such as mitosis and cell growth, signal transduction, and the translation and transcription of mRNA (Krebs et al., 1988; Pinna, 1990; Peppercock et al., 1991). Consistent with the diverse functions of CK II, its proposed physiological substrates are quite varied. They include at least eight different metabolic enzymes (Tuazon and Traugh, 1991), cytoskeletal proteins (Diaz-Nido et al., 1988), transcription factors (Berberich and Cole, 1992; Klimczak et al., 1992), calmodulin (Sacks et al., 1992), and nucleoskeletal lamin-like proteins (Li and Roux, 1992b).

The CK II kinases themselves are substrates for phosphorylation by $p34^{cdc2}$ (Litchfield et al., 1992) and protein kinase C (Sanghera et al., 1992). The M-phase-specific $p34^{cdc2}$ protein kinase can phosphorylate the β subunit of CK II and increase its activity (Mulner-Lorillon et al., 1990), and when epidermal growth factor stimulates CK II activity, it apparently does so by stimulating an increase in its phosphorylation state (Ackerman et al., 1990). These findings indicate that CK II is likely to be part of a protein kinase cascade.

Biochemical studies on CK II isolated from most organisms have revealed heterotetramers of the structure $\alpha_2\beta_2$ or $\alpha\alpha'\beta_2$ (Hathaway and Traugh, 1982; Edelman et al., 1987; Pinna, 1990). The two larger subunits, α and α', usually have molecular masses between 36 and 44 kD; the β subunit is typically between 25 and 30 kD, although in yeast it is 41 kD (Pinna, 1990). The α and α' subunits provide the catalytic activity, but the role of the β subunit remains poorly defined. Current evidence indicates that the β subunit can stimulate the activity of the holoenzyme, and thus presumably plays a regulatory role (Lin et al., 1991). Protein kinases with biochemical properties closely resembling those of CK II, but apparently lacking the typical heterotetrameric structure of animal and yeast CK II, have been described in plants (Erdmann et al., 1982; Godwa and Pillay, 1982; Yan and Tao, 1982; Erdmann et al., 1985; Dobrowolska et al., 1987). Recently, Li and Roux (1992a) described the properties of a heterotetrameric CK II kinase purified from a chromatin-enriched fraction of pea nuclei. Other characteristic biochemical properties of CK II enzymes are that they are typically activated by polyamines and inhibited by low concentrations of heparin and can utilize GTP equally as well as ATP as a phosphoryl donor substrate (Pinna, 1990).

In animal cells CK II protein kinases are predominately nuclear enzymes (Krek et al., 1992). Consistent with this finding, our laboratory has reported on two CK II kinases in pea (*Pisum sativum* L.) nuclei (Li and Roux, 1992a, 1992b). However, when we tested the cytoplasmic fraction from extracted pea plumules, we found evidence for significant CK II activity there. Here we report the purification to near homogeneity of a CK II-type protein kinase from the cytoplasmic fraction of etiolated pea plumules, and we document both that its biochemical properties are distinct from those of the CK II kinases of pea nuclei and that its activity may be

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Abbreviations: A_{50}, concentration of reagent needed to stimulate enzyme activity to 50% of the maximal activity; CK, casein kinase; DiCl-RB, 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole; I_{50}, concentration of reagent needed to inhibit enzyme activity by 50%.

regulated by autophosphorylation and dephosphorylation in vitro.

MATERIALS AND METHODS

Plant Growth

Seedlings of pea (*Pisum sativum* L. cv Alaska) were grown in the dark for 7 d at 23 ± 3°C.

Chemicals

Percoll, ATP, PMSF, DTT, aprotinin (bovine lung), DiCl-RB, DL-phosphoserine, DL-phosphothreonine, DL-phosphotyrosine, dephosphorylated casein, dephosphorylated α-casein, dephosphorylated β-casein, phosvitin, calf thymus histone III-S, myosin light chains (bovine muscle), poly-L-Lys (degree of polymerization = 60), poly-L-Arg (degree of polymerization = 60), poly-L-Glu (degree of polymerization = 70), cellulose phosphate (medium mesh), casein agarose, and protamine chloride (salmon) were all obtained from Sigma. The [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from New England Nuclear. Alkaline phosphatase bound to beaded agarose was obtained from Sigma. All buffers were prepared with water purified by a Milli Q water purification system (Millipore Corp.).

Preparation of Cytosolic Fraction

Pea plumules (100 g) were harvested, immersed in cold diethyl ether for 2 min, air-dried on Whatman filter paper, and then homogenized in buffer A (1 M Suc, 10 mM Hepes, 5 mM MgCl$_2$, and 10 mM β-mercaptoethanol, pH 7.0) with a Polytron (PTA 20ts, Brinkman) at setting 3 for 30 to 40 s and setting 2.5 for 1 to 2 min. The homogenate was filtered through three layers of Miracloth and one layer each of 80-μm and 30-μm nylon mesh, and then centrifuged at 4,500 rpm in a JA-20 rotor for 10 min to remove nuclei, large debris, and unbroken cells. The supernatant fraction was adjusted to 50 mM NaCl, 0.1 mM PMSF, 1 mL/L of aprotinin, 1 mM EDTA and centrifuged again for 2 h at 15,000 rpm in a JA-17 rotor. The supernatant from this centrifugation was considered the cytosolic fraction.

Purification of CK II

All purification procedures were carried out in a 4°C chamber. The cytosolic fraction was loaded onto a DEAE-Trisacryl (M-type, IBF Biotechnics, Columbia, MD) column (2.5 × 12 cm) preequilibrated with buffer B (50 mM Tris, pH 7.5, 1 mM EDTA, 2 mM DTT, 0.1 mM PMSF, 1 mL/L of aprotinin, 10% glycerol [v/v]) plus 50 mM NaCl. The column was eluted with a linear gradient (300 mL) of NaCl from 50 to 500 mM in buffer B at approximately 2 mL/min after being washed with 250 mL of buffer B plus 50 mM NaCl. The activity was eluted as a sharp peak at 200 mM NaCl (Fig. 1A). The active fractions were pooled and loaded onto a cellulose phosphate column (1.5 × 25 cm) that had been equilibrated with buffer B plus 250 mM NaCl. After the column was washed with 3 column volumes of equilibration buffer, it was developed with a 300-mL NaCl gradient (250–1000 mM)
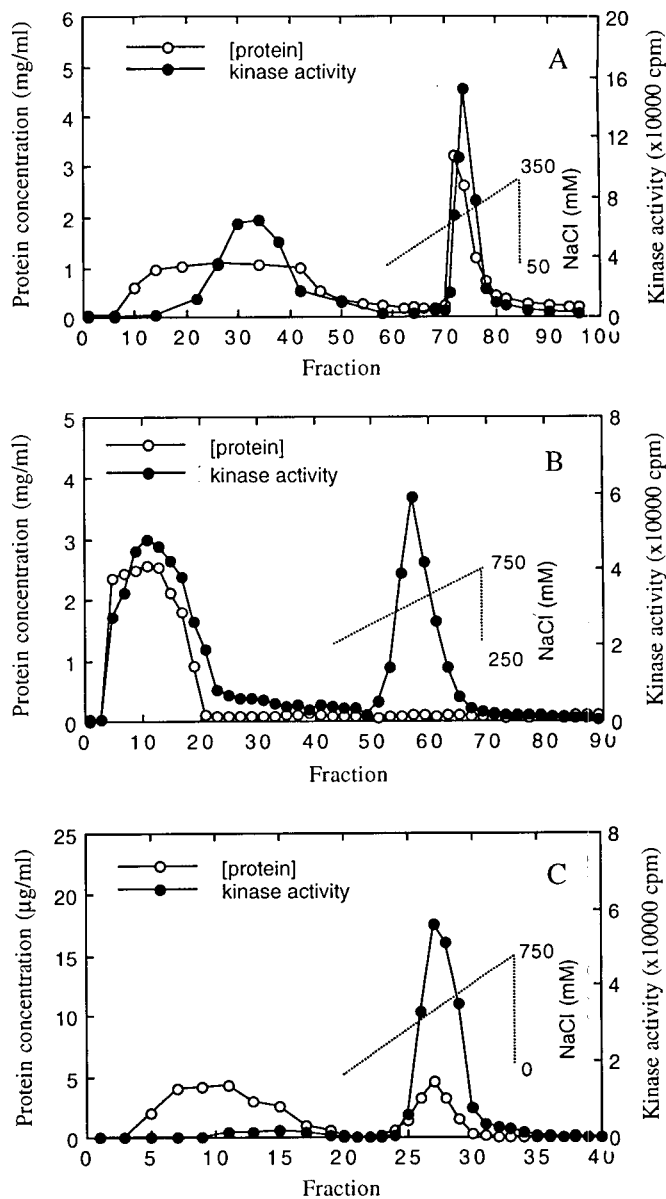
Figure 1. Elution profiles of protein concentration and kinase activity from DEAE-Trisacryl (A), cellulose phosphate (B), and casein-agarose columns (C). The insets indicate the NaCl gradient profile.

in buffer B at approximately 2 mL/min. The activity was eluted at about 500 mM NaCl (Fig. 1B) and the active fractions were loaded onto a 1 × 7 cm hydroxylapatite (high resolution, CalBiochem) column preequilibrated in buffer B plus 500 mM NaCl. The column was washed with 3 column volumes of starting buffer, 3 volumes of buffer B, and 3 volumes of 25 mM potassium phosphate, pH 7.5, with 1 mM EDTA and 2 mM DTT. Activity was eluted with 300 mM potassium phosphate buffer. After overnight dialysis against buffer B plus 5 mM MgCl$_2$ in Spectra/Por 6 dialysis membrane (mol wt cutoff = 10,000), the pooled enzyme fraction was loaded onto a casein-agarose affinity column (1 × 7 cm) equilibrated with the same buffer. After being washed with 3 column volumes

of equilibration buffer, the column was eluted with an 80-mL linear NaCl gradient (0–500 mM) in equilibration buffer at approximately 0.2 mL/min. The activity was eluted at about 300 mM NaCl (Fig. 1C).

Molecular Sieve HPLC

Native mol wt of the kinase was determined by co-chromatography of the purified enzyme with mol wt standards (Bio-Rad) at room temperature using an Ultraspherogel SEC 3000 column (0.75 × 30 cm; Beckman Instruments) equilibrated in 50 mM phosphate buffer. The flow rate was 1 mL/min, and 0.2-mL fractions were collected.

Enzyme Assay

Protein kinase was assayed by measuring the incorporation of ^{32}P from $[\gamma\text{-}^{32}P]ATP$ into casein or β-casein following the method of Hathaway and Traugh (1983) with modification. The standard assay mixture (50 μL) contained 50 mM Tris, pH 8.0, 5 mM $MgCl_2$, 1 mg/mL of casein, 100 μM ATP (about 1000 cpm/pmol), and 10 μL of enzyme solution. The reaction was initiated by addition of ATP. Under standard assay conditions, the ^{32}P incorporated into dephosphorylated casein substrate increased linearly up to 45 min. After the assay mixtures were incubated at 25°C for 15 min, the reactions were terminated by spotting 30 μL of reaction mixture onto a P81 filter disc (Whatman) and submerging the filter in 75 mM H_3PO_4. The filters were washed for 10 min each in three changes of the same solution, then rinsed with ethanol for 1 min, followed by diethyl ether for 30 s, and air dried. Radioactivity on the filters was determined in 5 mL of scintillation fluid (Bio-Safe II, RPI Corp., Mount Prospect, IL) using a Packard 1500 Tri-Carb liquid scintillation analyzer. All kinetic studies were carried out with casein or β-casein as substrate. About 5 units of enzyme were used in each reaction, with 1 unit defined as the amount of enzyme that will catalyze the transfer of 1 pmol of phosphate from ATP to casein in 1 min.

SDS-PAGE and Densitometry

Electrophoresis in 6% or 12% acrylamide-SDS gels was carried out according to the method of Laemmli (1970). All samples for electrophoresis analysis were precipitated with 15% (w/v) TCA. The pellet was washed twice with ice-cold ethanol:diethyl ether (1:1, v/v) and air dried, and then the protein was dissolved in SDS sample buffer and boiled for 3 min. The gel was stained with Coomassie blue, destained, and dried between two layers of cellophane membrane. The density of each band was determined by absorbance scanning using a ISCO UA-5 absorbance/fluorescence detector.

Activity Gel

The in situ protein kinase activity was detected following SDS-PAGE according to the method of Guo and Roux (1990) with some modifications. The enzyme samples were added to SDS sample buffer without boiling and electrophoresed on SDS-polyacrylamide gels as described by Laemmli (1970) except that 1 mg/mL of casein was added to the running gel before polymerization. SDS was removed by washing the gel

immediately after electrophoresis with buffer C (50 mM Tris, pH 8.0, 2 mM DTT, 5 mM $MgCl_2$, 0.5% Triton X-100 [v/v], 1 mg/mL of BSA, and 250 mM NaCl) three times, each for 30 min at room temperature. The enzyme was allowed to renature in buffer B with 5 mM $MgCl_2$ at 4°C overnight after being washed in the same buffer twice. The gel was then incubated at room temperature overnight with gentle shaking in 20 mL of standard enzyme assay mixture except that the concentration of ATP was 2.5 nM (200 μCi). The reaction was stopped by the addition of 5% (w/v) TCA in 1% (w/v) NaPPi solution after removing the reaction mixture. The gel was washed extensively with the same solution, stained with Coomassie blue, and destained to visualize the size marker. For autoradiography, the dried gel was exposed to Kodak XAR-5 film at −70°C.

Autophosphorylation in Vitro

Autophosphorylation of the kinase was accomplished by incubating the purified enzyme preparation with $[\gamma\text{-}^{32}P]ATP$ at 25°C for various periods of time. The kinase activity was then assayed by addition of substrate as described above. The subunit that was autophosphorylated was identified by SDS-PAGE and autoradiography.

Assay of Phosphorylated Amino Acids in the Substrates

The procedure used was similar to that described by Putnam-Evans et al. (1990). Phosphorylated substrates was precipitated using 10% (w/v) TCA. After being washed with 10% (w/v) TCA and ethanol, the pellets were hydrolyzed in 6 N HCl for 2 h at 110°C, dried in a Speed-Vac evaporator, and then resuspended in 10 μL of phosphoamino acid standards (1 mg/mL each of DL-phosphoserine, DL-phosphothreonine, and DL-phosphotyrosine). The phosphoamino acids were separated by high-voltage paper electrophoresis (Shandon L24 high-voltage electrophoresis apparatus and Whatman No. 1 paper) in pyridine:acetic acid:water (5:50:945, v/v/v) at 3000 V for 45 min. The position of the standards was visualized by ninhydrin (0.2% [w/v] in acetone), and the labeled amino acids were detected by autoradiography.

Alkaline Phosphatase Pretreatment of CK II in Vitro

Alkaline phosphatase-beaded agarose (Sigma) was washed three times in water and resuspended in reaction buffer (50 mM Tris, pH 8.0, 0.5 mM $ZnCl_2$, 5 mM $MgCl_2$). One unit of phosphatase activity was added to 50 μL of CK II solution and incubated at room temperature for various times. The reaction was stopped by centrifugation to remove the phosphatase beads. The supernatant fractions were analyzed for CK II activity as described. The phosphatase inhibitors NaF (10 mM) and $(NH_4)_6Mo_7O_{24}$ (0.2 mM) were included in all assay mixtures to inactivate any alkaline phosphatase that may have been cleaved from agarose beads during the pretreatment period.

Protein Determination

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

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Table I. *Purification of CK II protein kinase from the cytosolic fraction of pea plumules*

Fraction	Protein	Total Activity	Specific Activity	Recovery[a]	Purification
	mg	nmol min⁻¹	nmol min⁻¹ mg⁻¹	%	fold
Cytosolic fraction	1053.00	41.00	0.04	100	1
DEAE-Trisacryl	267.00	42.70	0.16	102	4
Cellulose phosphate	0.25	7.10	28.40	17	710
Hydroxylapatite	0.15	5.48	36.53	13	913
Casein-agarose	0.01	3.28	328.00	8	8200

[a] The starting cytosolic fraction was prepared from 100 g of pea plumules.

RESULTS

### Purification of Protein Kinase

A typical purification of CK II from the cytosolic fraction of pea plumules is summarized in Table I, with typical elution profiles from the column chromatography steps shown in Figure 1. Analysis by SDS-PAGE revealed that the final product consisted of three major polypeptides (designated α, α', and β) and a small number of minor contaminants (Fig. 2A). The three major polypeptides, but not the contaminants, co-purified with the activity at each step in the purification protocol. As estimated by densitometry of Coomassie blue-stained gels, α, α', and β together constituted about 90% of the protein in the purest fraction. More than 8000-fold puri-

fication was required to obtain enzyme of this purity (Table I). This result indicated that the CK II enzyme that was purified was approximately 0.01% of the total soluble protein in the cytosolic fraction.

Mol Wt and Subunit Composition

The molecular masses of the α, α', and β subunits are 41, 40, and 35 kD, respectively (Fig. 2A and Table II). The apparent stoichiometry of the three polypeptides, as estimated by densitometry of gels stained with Coomassie blue, was about 0.7:1.1:2.0 (α:α':β). An $\alpha\alpha'\beta_2$ structure would have a predicted molecular mass close to the value calculated from HPLC-based size-exclusive chromatography, about 150 kD (Table II). The activity gel revealed two ³²P-labeled bands around 40 and 41 kD on the autoradiograph (Fig. 2B), indicating that probably both the α and α' subunits retained activity after SDS-PAGE and in situ renaturation.

Incubation of the purified kinase with [γ-³²P]ATP in the absence of added substrate resulted in the incorporation of ³²P into the enzyme. Analysis of the autophosphorylated enzyme by SDS-PAGE and autoradiography revealed that most of the label was incorporated into the β subunit (Fig. 2C).
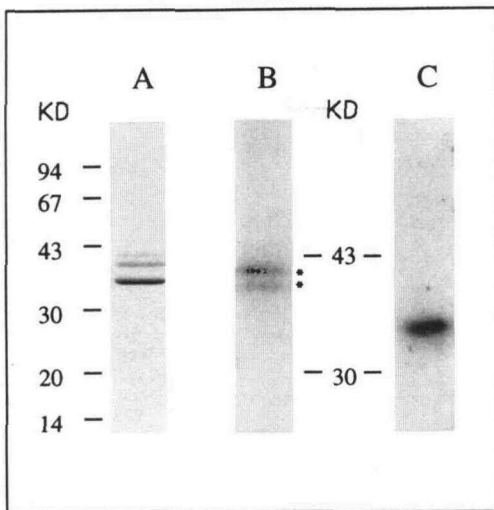
Figure 2. A, Peak fraction of CK II protein kinase activity from the casein-agarose column after 12% SDS-PAGE and Coomassie blue staining, showing three kinds of subunits with a ratio of approximately 0.7:1.1:2.0. Total protein loaded was about 5 µg. B, Autoradiogram showing the catalytic subunits. After being separated on a 6% SDS-polyacrylamide gel embedded with casein, the enzyme (20 units) was renatured in situ and incubated with [γ-³²P]ATP as described in "Materials and Methods." After unbound [γ-³²P]ATP was washed out, the gel was dried and analyzed by autoradiography. Asterisks indicate two catalytic subunits. C, Autoradiogram showing that the β subunit can be autophosphorylated. Ten units (about 30 ng) of enzyme was incubated with [γ-³²P]ATP (100 µM at 5000 cpm/pmol) for 15 min. After SDS-PAGE, the dried gel was analyzed by autoradiography.

Table II. *Structural and kinetic properties of CK II*

Parameter	Value
Structural parameters	
Native mol wt[a]	150,000
Subunit composition[b]	
α-subunit	41,000
α'-subunit	40,000
β-subunit	35,000
$\alpha\alpha'\beta_2$ tetramer, calculated	151,000
Kinetic parameters	
Mg²⁺-ATP	
K_m (µM)	50
V_{max} (µmol min⁻¹ mg⁻¹)	0.68
Mg²⁺-GTP	
K_m (µM)	60
V_{max} (µmol min⁻¹ mg⁻¹)	0.50

[a] Measured relative to internal standards using a Ultraspherogel SEC 3000 column.　　[b] Mol wts measured relative to known standards by SDS-PAGE.
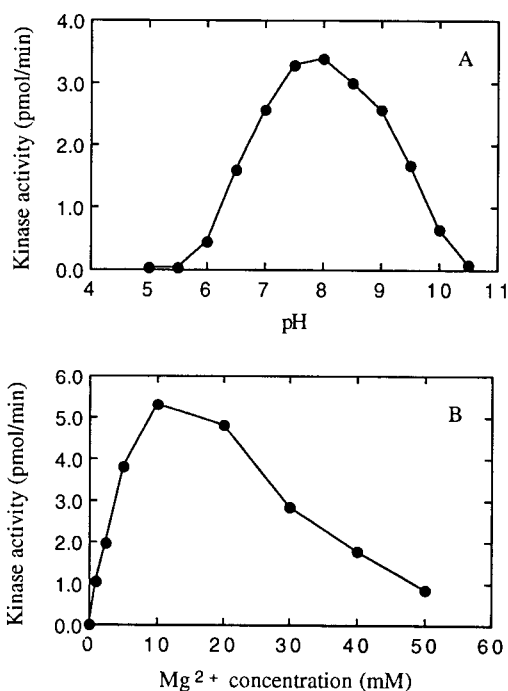
Ionic Requirements for Activity

Optimum pH for the purified CK II enzyme was 8.0. Half-maximal activity was observed at pH 6.5 and pH 9.5 (Fig. 3A). The enzyme exhibited an absolute requirement for Mg^{2+}. Activity was strongly stimulated by $MgCl_2$ up to a total concentration of 10 mM (Fig. 3B). Stimulation by Mg^{2+} in excess of the amount necessary for formation of a stable Mg-ATP^{2-} complex suggests an additional function(s) for this metal ion. Beyond 10 mM $MgCl_2$, an inhibition of activity was observed. In contrast to pea nuclear CK II reported by Li and Roux (1992a) and most other CK IIs, the cytosolic CK II was inhibited by K^+ and Na^+, with I_{50} values of 75 and 65 mM, respectively (Table III). At 200 mM K^+ or Na^+, activity was inhibited by more than 90%. This property is also shared by CK II from yeast (Meggio et al., 1986).

Substrate Specificity

The purified enzyme was able to use Mg-GTP^{2-} almost as efficiently as Mg-ATP^{2-} as a phosphate donor. At 1 mg/mL casein, the enzyme exhibited an apparent K_m and V_{max} for Mg-GTP^{2-} of 60 μM and 0.50 μmol min^{-1} mg^{-1}, respectively, compared to 50 μM and 0.68 μmol min^{-1} mg^{-1}, respectively, for Mg-ATP^{2-} under the same conditions (Table II).

Of the proteins tested for their ability to serve as exogenous substrates, only casein (mixed, α, and β) and phosvitin proved

Table III. Effects of various reagents on CK II kinase activity

Values represent the mean of triplicate readings. SD in all cases was ≤10% of the mean value. The assay conditions were as described in "Materials and Methods," using ATP as the phosphoryl donor.

Reagent	Concentration	Relative Activity
		% of control
Basic polypeptides		
poly-L-Lys	5.0 μg/mL	123
	60 μg/mL	50
poly-L-Arg	5.0 μg/mL	126
	60 μg/mL	50
protamine	2.5 μg/mL	128
	85 μg/mL	50
NaCl	65 mM	50
KCl	75 mM	50
PO_4^{3-}	3.5 mM	50
DiCl-RB	0.07 mM	50
poly-L-Glu	1 μg/mL	50

to be good acceptors (Table IV). The apparent K_m for phosvitin (0.02 mg/mL) was considerably lower than that observed for casein (0.29, 0.19, and 1.30 mg/mL for mixed, α-, and β-casein, respectively). However, the apparent V_{max} with phosvitin as substrate was much lower (0.13 μmol min^{-1} mg^{-1} compared to 0.50, 0.41, and 0.68 μmol min^{-1} mg^{-1} for mixed, α-, and β-casein, respectively). Histone III-S was a poor substrate, and muscle myosin light chains and BSA were not substrates (Table IV).

Both Ser and Thr residues in mixed casein, β-casein, and phosvitin could be phosphorylated, whereas only Ser was phosphorylated when using α-casein or histone III-S as substrate (data not shown). No phosphorylation of Tyr residues was observed.

Effectors of CK II Activity

Under standard assay conditions, the kinase activity was stimulated 130% by 0.5 mM spermine (A_{50} = 0.1 mM) and 80% by 2.5 mM spermidine (A_{50} = 0.4 mM), whereas putrescine and cadaverine had no stimulatory effect up to 10 mM (Fig. 4A).

The kinase purified from the pea cytosolic fraction was

Figure 3. Effects of pH and Mg^{2+} on CK II kinase activity. About 5 units of enzyme were used in each reaction. A, Effect of pH. Buffers used were 50 mM Mes (pH 5.0–6.50), 50 mM Tris (pH 6.5–9.0), and 50 mM 3-[cyclohexylamino]-1-propanesulfonic acid (Caps, pH 9.0–10.5). B, Effect of $MgCl_2$. The kinase preparation used was first dialyzed against 1000 mL of buffer B without $MgCl_2$ for 24 h with three changes at 4°C.

Table IV. Comparison of CK II protein substrates

Values were derived from reciprocal plots of data obtained under the assay conditions described in "Materials and Methods," using ATP as the phosphoryl donor.

Substrate	K_m	V_{max}
	$mg\ mL^{-1}$	$\times 10^5\ pmol\ min^{-1}\ mg^{-1}$
Casein	0.29	5.00
α-Casein	0.19	4.10
β-Casein	1.30	6.79
Phosvitin	0.02	1.30
Histone III-S	0.88	0.70
Muscle myosin light chains	0.14	0.22
BSA	0.04	0.05
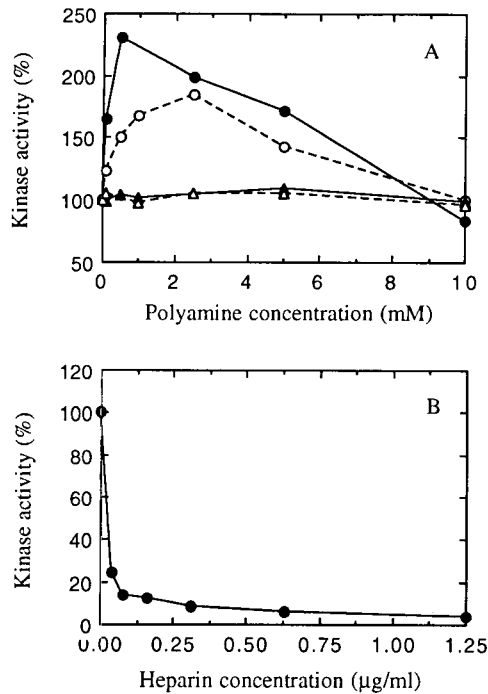
Figure 4. Effect of different polyamines [A, Spermine (●), spermidine (○), putrescine (▲), cadaverine (△). B, Heparin] on the CK II kinase activity. Initial velocity in the absence of reagent (100% activity) was about 0.5 μmol min^{-1} mg^{-1}.

very sensitive to heparin (I_{50} = 0.025 μg/mL); 0.3 μg/mL inhibited its activity by 90% (Fig. 4B). This is one of the diagnostic characteristics of CK II.

Using casein as an artificial substrate, the I_{50} of the purified kinase by a variety of other inhibitors and the maximal stimulation by some stimulators was determined (Table III). Phosphate, DiCl-RB, and poly-L-Glu, which have been shown to be effective inhibitors of animal CK II (Meggio et al., 1990; Pinna, 1990), are also very effective inhibitors of the pea cytosolic CK II kinase. Only minor stimulation was obtained by addition of low concentrations of poly-L-Lys, poly-L-Arg, or protamine into the reaction mixture. At higher concentrations, the activity was inhibited with I_{50} values of 60, 60, and 85 μg/mL for poly-L-Lys, poly-L-Arg, and protamine, respectively.

Regulation of Kinase Activity

Activity of the purified enzyme was unaffected by cAMP, cGMP, and Ca^{2+}-EGTA (data not shown). After pretreatment with beaded alkaline phosphatase, the CK II activity decreased nearly 5-fold (Fig. 5A). Addition of NaF and (NH$_4$)$_6$Mo$_7$O$_{24}$ to the phosphatase reaction mixture totally blocked phosphatase activity and eliminated this effect. As an additional control, the phosphatase beads were incubated at room temperature for 2 h in the same buffer that was used for the phosphatase-CK II reaction. The beads were then pelleted by centrifugation and the supernatant was added to the kinase assay mixture at the same time as CK II addition. The supernatant had no effect on the final cpm incorporated

into casein; thus, it is unlikely that it caused the release of ^{32}P from either casein or lowered the concentration of [γ-^{32}P] ATP by hydrolyzing it. Analysis by SDS-PAGE showed that the banding patterns of the enzyme preparation before and after phosphatase treatment were identical. This indicated that the decrease in kinase activity was not due a protease activity that may have possibly existed in the phosphatase preparation. Attempts to restore CK II activity by subsequent phosphorylation with another protein kinase, including a CK I-type kinase (data not shown) and a calcium-dependent kinase (Li et al., 1991) from pea, were unsuccessful.

Incubation of the kinase preparation with ATP for as little as 5 min in the absence of an exogenous protein substrate resulted in the autophosphorylation of the β subunit and the simultaneous abolition of kinase activity (Fig. 5B), suggesting an inverse relationship between autophosphorylation and kinase activity. This autophosphorylation and activity inhibition does not occur in the presence of casein, since the
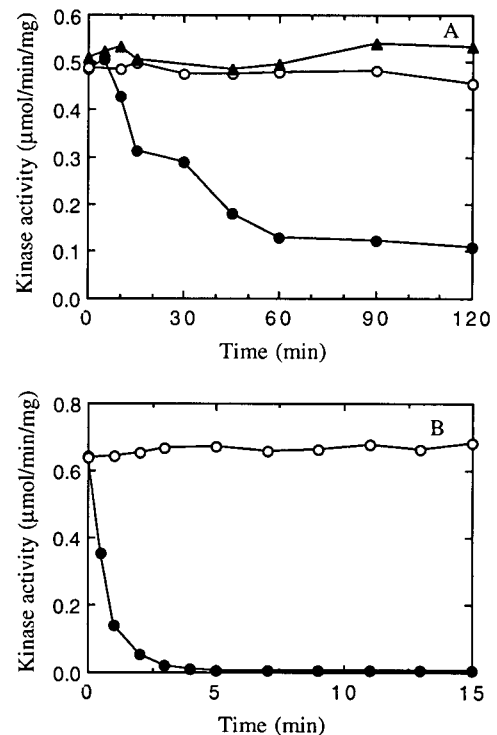
Figure 5. A, Effects of pretreatment with beaded alkaline phosphatase on the kinase activity (●). Two controls were activity assayed after adding together with 10 mM NaF and 0.2 mM (NH$_4$)$_6$Mo$_7$O$_{24}$ into the reaction mixture (○), and activity assayed without phosphatase (▲). At the times indicated, insoluble phosphatase was removed by centrifugation and aliquots (10 μL each) of kinase were taken out and assayed as described in "Materials and Methods"; all final assay mixtures contained 10 mM NaF and 0.2 mM (NH$_4$)$_6$Mo$_7$O$_{24}$. B, Effects of autophosphorylation on the kinase activity. Enzyme preparation was incubated with 500 μM [γ-^{32}P]ATP (1000 cpm/pmol), and at times indicated aliquots (10 μL each) were taken out and assayed under standard condition (●). The control experiment was done side by side; the only difference was that the ATP and enzyme were added to the assay mixture at the same time (○).

incorporation of ^{32}P into casein increases linearly over time for up to 45 min (data not shown).

DISCUSSION

The pea cytosolic CK II described here is closely related to the CK II-type kinase from pea nuclei described by Li and Roux (1992a). According to the criteria reviewed recently by Pinna (1990), both are typical CK II-type kinases in their: (a) native mol wts; (b) heterotetrameric quaternary structure; (c) ability to use both ATP and GTP as phosphoryl donor; (d) stimulation by polyamines; (e) high sensitivity to inhibition by heparin; (f) preference for casein and phosvitin as artificial substrate; (g) selective phosphorylation of Ser and Thr side chains; (h) independence of known second messengers for their activity; and (i) kinetic (K_m, V_{max}) properties. However, these two pea kinases also differ from one another in several aspects. The cytosolic CK II has larger subunits and a larger native molecular mass (150 kD versus 130 kD for nuclear CK II), two different catalytic subunits ($\alpha\alpha'$) instead of the α_2 catalytic subunits of pea nuclear CK II, and a higher sensitivity to heparin inhibition (I_{50} value of 0.025 $\mu g/mL$ versus I_{50} value of 0.1 $\mu g/mL$ for nuclear CK II). Also, the cytosolic CK II kinase is almost completely inhibited by 0.2 M NaCl or KCl, whereas the nuclear kinase is stimulated by the same concentration of these salts.

Both $\alpha_2\beta_2$ and $\alpha\alpha'\beta_2$ heterotetramers are commonly found among the characterized CK II kinases (Pinna, 1990). Based only on the intensity of Coomassie blue staining, the amount of the 41-kD subunit (α) of the CK II kinase appears to be less than that of the 40-kD subunit (α') (Fig. 2A). However, the activity gel reveals that the α and the α' subunits have approximately the same amount of protein kinase activity (Fig. 2B). One interpretation of this apparent discrepancy is that the two different catalytic subunits have different staining affinities for Coomassie blue. There is precedent for such unequal staining in the two catalytic subunits of other CK II molecules (Tuazon and Traugh, 1991). Alternatively, perhaps only some molecules of CK II have an $\alpha\alpha'\beta_2$ quaternary structure, whereas others have two 40-kD catalytic subunits.

The purification of both the 150-kD cytosolic CK II and the 130-kD nuclear CK II uses DEAE chromatography as an early purification step. Based on our observation that after this step little or no 150-kD CK II can be found in nuclear extracts, nor can 130-kD CK II be found in cytosolic extracts, we judge that these two kinases usually reside in two different subcellular compartments. However, in bovine adrenocortical cells there is a significant shift of the intracellular distribution of CK II kinase from the cytoplasm into the nucleus during active proliferation of these cells in culture, and this process is stimulated by polyamines (Filhol et al., 1990). In plants, it is well documented that the polyamine level in the cells increases before the onset of cell division (Smith, 1985; Galston and Sawhney, 1990), so the possibility of regulated exchange between cytoplasmic and nuclear CK II should be considered in the plant cell as well.

One possible regulation mechanism for CK II in animal cells is (auto)phosphorylation (Pinna, 1990). Meggio and Pinna (1984) have reported that autophosphorylation of a rat liver cytosol CK II kinase inhibits its activity, whereas Mulner-

Lorillon et al. (1990) have reported that the phosphorylation of a frog CK II kinase by p34^{cdc2} protein kinase in vitro stimulates its activity about 2.5-fold. Consistent with these reports, the pea CK II kinase is inhibited by autophosphorylation, and maintenance of the phosphorylation of a presumably different site is also critical for its activity, since treatment by alkaline phosphatase can block its capacity to phosphorylate casein. Since we have been unable to label the CK II kinase with ^{32}P by treatment with other kinases, we have no direct evidence that the alkaline phosphatase treatment is actually dephosphorylating a site on the kinase. However, the observations that phosphatase inhibitors block the inhibitory effects of the alkaline phosphatase and no proteolysis occurs during the phosphatase treatment both support the interpretation that the effects of alkaline phosphatase are indeed due to its dephosphorylation of the kinase. This interpretation must be considered only probable until a more direct demonstration of this dephosphorylation is achieved.

Light stimulates an increase in the Arg decarboxylase activity and polyamine content of bud tissue in etiolated pea seedlings, and this process is mediated by phytochrome (Dai and Galston, 1981). Since the level of spermine required to obtain complete stimulation of pea cytosolic CK II (0.5 mM) is well within the physiological range of polyamine levels in plants, this kinase could be one of the targets of polyamine action in the process of light signal transduction (Roux, 1993). However, not enough is known about either the concentration of polyamines in specific subcellular compartments or how rapidly this concentration can be modulated by physiological agonists to predict whether CK II kinases are important transducers of polyamine effects in plant cells.

CK II-type protein kinases have been implicated in the regulation of light-stimulated transcription. The trans-acting factors AT-1 and GBF1 both bind to promoter regions of light-regulated genes, and the DNA-binding activities of both are reversibly modulated by phosphorylation catalyzed by a CK II-type kinase (Datta and Cashmore, 1989; Klimczak et al., 1992). In principle, the site of factor phosphorylation could be cytosolic or nuclear, so CK II kinases in either locale are potential candidates for this function. As discussed above, the predominantly cytoplasmic locale of the CK II kinase described here does not exclude the possibility of it being transiently moved into the nucleus and functioning there.

In human fibroblast cells, blocking out the function of a CK II kinase has been accomplished by incorporating antisense oligodeoxynucleotides complementary to the translation start region of the mRNAs encoding the CK II α and β subunits. These experiments have demonstrated the critical role of this kinase in cell-growth stimulation by epidermal growth factor (Pepperkok et al., 1991). Purification of the cytoplasmic CK II kinase in pea has laid the groundwork for obtaining primary sequence data on this enzyme and raising antibodies to it. These advances will facilitate the kind of antisense experiments that will ultimately be needed to discover what cellular processes are controlled by this unique CK II kinase.

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