Characterization of a winged bean (*Psophocarpus tetragonolobus*) protein kinase with calmodulin-like domain: regulation by autophosphorylation

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A soluble protein kinase purified from winged bean (*Psophocarpus tetragonolobus*) shoots, has been assessed as a monomeric enzyme with an approximate M_r of 60000 in spite of the presence of two polypeptides of 61 and 58 kDa determined by SDS/PAGE. Immunoblot analyses using either of the two antisera raised individually against the polypeptides, detect both of them in purified preparations and a single larger polypeptide (62 kDa) in freshly prepared tissue homogenates, clearly indicating the likelihood of the doublet being formed from the larger one by proteolysis. Histone H1, syntide 2 and a synthetic myosin light chain-related peptide (MLC-peptide) have been identified as exogenous substrates of the enzyme. Complete Ca^{2+}-dependence for substrate phosphorylation, a drastic inhibition of the reaction by a calmodulin (CaM) antagonist which can be partially reversed by a heterologous CaM and direct $^{45}Ca^{2+}$-binding on blot, form compelling evidence in favour of a CaM-like domain of the enzyme. Both the polypeptides of the purified enzyme undergo intramolecular autophosphorylation on serine residue(s). Unlike the substrate phosphorylation reaction, autophosphorylation is Ca^{2+}-independent and is not inhibited by the CaM antagonist. Down-regulation of substrate phosphorylation by autophosphorylation, and stimulation of the autophosphorylation by histone H1 and MLC-peptide, are novel regulatory features of the enzyme.

INTRODUCTION

A central role of Ca^{2+} in a variety of stimulus–response processes has been widely recognized for many eukaryotic cells [1,2]. An incoming signal causes the rise of the intracellular level of Ca^{2+} ion, modulating inactive Ca^{2+}/calmodulin (CaM)-dependent protein kinases (CaM kinases). The active enzymes then catalyse the phosphorylation of many proteins, some of which play key roles in diverse cellular processes [3–5]. The functional properties of such proteins, serving as substrates of these enzymes, are often dependent on their state of phosphorylation and thus protein kinases act as pleiotropic regulators for many cellular functions. Furthermore, protein kinases are themselves frequently regulated by phosphorylation [6–8].

Several protein kinases have been purified and characterized and discovery of new ones in the expanding protein kinase family of enzymes continues. While the Ca^{2+}/calmodulin-dependent and the Ca^{2+}/lipid-dependent protein kinases are known to occur commonly in animal cells [9,10], a new class of protein kinase has been recently recognized [11] and a few of them have been characterized in plants [12–14] and a ciliated protozoan [15,16]. The newly discovered enzyme is dependent on Ca^{2+}, but independent of CaM for activity, and the unique structural feature, distinguishing it from other Ca^{2+}-dependent enzymes like CaM kinases and protein kinase C, is the presence of a CaM-like, EF-hand motif in the C-terminal region of the polypeptide chain [11]. This Ca^{2+}-binding domain is covalently linked to the N-terminal part having the catalytic domain by a junction sequence. The presence of a putative auto-inhibitory sequence in the junction has also been suggested for this novel class of protein kinase [13,14]. While most workers differentiate it from other protein kinases by referring to it as the 'Ca^{2+}-dependent protein kinase' (CDPK), Roberts [13] seems to prefer the term 'calmodulin-like domain protein kinase' (CDPK). With the recent recognition of the soybean enzyme to be the first member of this distinct class of protein kinases [17], other CDPKs have also been isolated from oat [18], *Arabidopsis thaliana* [19], groundnut [20] and *Paramecium tetraurelia* [16].

Like other protein kinases, CDPKs have also been shown to undergo autophosphorylation [16,17,20,21] although much is not known regarding the control of autophosphorylation of CDPKs and its possible role in the regulation of substrate phosphorylation. In the present studies, we describe the isolation and characterization of another CDPK from winged bean (*Psophocarpus tetragonolobus*). It undergoes autophosphorylation even in the presence of calmodulin antagonists, like fluphenazine, and the absence of Ca^{2+} and the autophosphorylation leads to down-regulation of substrate phosphorylation.

MATERIALS AND METHODS

Chemicals

$H_3{}^{32}PO_4$ and $^{45}CaCl_2$ were obtained from Bhabha Atomic Research Centre, India. [γ-^{32}P]ATP was prepared according to Glynn and Chappell [22]. Bovine brain CaM, fluphenazine, histone IIS and syntide-2 (PLARTLSVAGLPGKK) were purchased from Sigma, U.S.A. Smooth muscle myosin light chain-related peptide (MLC-peptide) (KKRPGRATSNVFS) and histone H1 were obtained from Peninsula Lab., Belmont, U.S.A. and GIBCO-BRL, U.S.A., respectively. Histone-Sepharose 4B was prepared according to Wilchek et al. [23]. Other chemicals used were analytical grade reagents.

Abbreviations used: MLC-peptide, smooth msucle myosin light chain-related peptide; CaM, calmodulin; CaM kinase, Ca^{2+}/calmodulin-dependent kinase; CDPK, calmodulin-like domain protein kinase; WbCDPK, winged bean calmodulin-like domain protein kinase.

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Plant tissue

Winged bean (*Psophocarpus tetragonolobus*) seeds were collected from the plants grown in the Institute garden and germinated at 25 °C in the dark. Shoots (4–5 days old) were used as the starting material.

Protein kinase assay

Protein kinase assay was routinely carried out by the P81 paper (Whatman)-binding method. The reaction mixture, in a total volume of 50 μl, contained 50 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol, 0.05 mM sodium vanadate, BSA (0.5 mg/ml), 0.05 mM [γ-^{32}P]ATP (specific activity 500–2000 c.p.m./pmol) and 0.05 mM MLC-peptide. The reaction was initiated by the addition of 0.5–1.0 μg of enzyme in 10 μl and incubation at 25 °C was done for a fixed time. The reaction was stopped by the addition of glacial acetic acid to a final concentration of 45%. Portions of the acidified reaction mixture were spotted onto P81 strips (2 cm × 2 cm) and the strips were washed three times in 15% acetic acid and then in 90% ethanol, dried and counted in a liquid scintillation counter.

Alternatively, the reaction was stopped by the addition of SDS sample buffer [24] and the mixture was boiled and then subjected to SDS/PAGE (20% acrylamide), followed by autoradiography. The phosphorylated protein was excised and ^{32}P incorporated into substrates was determined by liquid scintillation counting.

Purification of winged bean protein kinase

Winged bean shoots (85 g) were homogenized in 3 vol. of cold buffer A (50 mM Tris/HCl, pH 8.0/2 mM 2-mercaptoethanol), containing 1.5 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 5 mM benzamidine/HCl, 0.4% (v/v) fresh rabbit serum (in which the absence of protein kinase activity had been ascertained by assay), and polyvinylpyrrolidone (10% of tissue by wt.). The use of rabbit serum, as a source of α_2-macroglobulin, a potent non-specific protease inhibitor, had proved to be quite effective in the prevention of proteolysis [25]. The 100000 *g* supernate was fractionated on a DEAE-cellulose (Pierce) column, equilibrated with buffer A, the active enzyme being eluted by a linear gradient of NaCl (0–0.6 M) in buffer A. After dialysis it was further fractionated on a hydroxyapatite (Bio-Rad) column, equilibrated with the same buffer, the enzyme being eluted by a linear gradient of K_3PO_4 (25–300 mM) in buffer A. Finally, the purified winged bean protein kinase was obtained from a histone-Sepharose affinity column by elution with a linear gradient of NaCl (0–0.5 M).

Other methods

Analytical gel filtration [26] and sucrose density gradient ultracentrifugation [27] were carried out for the determination of the molecular mass of the purified enzyme. Polyclonal antibodies were raised against the individual polypeptides separated by SDS/PAGE [28] and used in immunoblot analysis [29,30].

Protein was estimated by the Coomassie Blue dye-binding method according to Bradford [31], using BSA as the standard. SDS/PAGE was carried out by the method of Laemmli [24].

RESULTS

Purification and characterization of a winged bean protein kinase

A protein kinase has been purified from the soluble extract of winged bean shoots by employing DEAE-cellulose, hydroxyapatite and histone-Sepharose column chromatography. Starting with 85 g of tissue, an approximate yield of 450 μg of purified enzyme is obtained, corresponding to a recovery of about 2% of the total protein kinase activity (Table 1).

Table 1 Purification of the winged bean protein kinase

The Table represents the results from a typical purification procedure starting with 85 g of tissue. Protein kinase assays were done by the P81-binding method using MLC-peptide as the substrate

Step	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min · mg)	Recovery (%)
1. 100000 *g* supernate	191.2	43.9	0.23	100
2. DEAE-cellulose	50.4	43.2	0.86	98.4
3. Hydroxyapatite	10.8	11.0	1.02	25.0
4. Histone-Sepharose	0.47	0.84	1.80	1.9

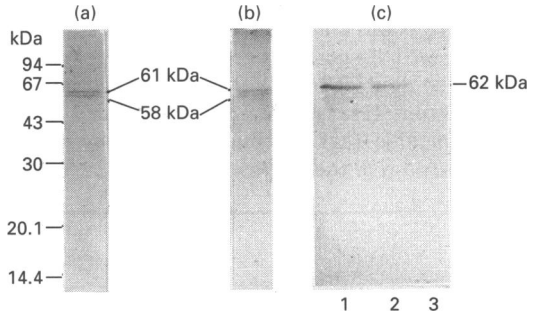

Figure 1 SDS/PAGE and immunoblot analysis of the winged bean protein kinase

(**a**) SDS/PAGE. The purified kinase preparation (2 μg of protein) was analysed by SDS/PAGE (12% acrylamide) and Coomassie Blue R-250 staining. (**b**) Immunoblot of the purified kinase preparation (1 μg of protein). (**c**) Immunoblot of the tissue extracts. Lanes: 1, crude homogenate (15 μg of protein); 2, 100000 *g* supernate (10 μg); 3, particulate fraction (5 μg). The numbers on the left margin indicate the molecular mass of the standards. The immunoblot analysis was carried out with the immune serum raised against the 61 kDa protein using protein A-alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate and Nitroblue Tetrazolium [30].

SDS/PAGE and immunoblot analysis show that the purified preparation is composed of two major polypeptides of 61 and 58 kDa, both of which are recognized by the same antiserum (Figures 1a and 1b). However, a single immuno-stained protein band (62 kDa) is detectable in the freshly prepared crude tissue extract as well as the 100000 *g* supernate, but none in the particulate fraction (Figure 1c, lanes 1, 2 and 3). All the three polypeptides (62, 61 and 58 kDa) are recognized by either of the two antisera raised against the 61 and 58 kDa polypeptides.

From the results of gel permeation chromatography and sucrose density gradient ultracentrifugation studies, an approximate M_r of 60000 has been estimated for the enzyme (results not shown).

Substrate specificity and autophosphorylation of the winged bean protein kinase

Among the various exogenous proteins/peptides tested, casein, protamine, angiotensin and Kemptide are not phosphorylated by the enzyme while histone H1, syntide 2 and MLC-peptide are

Table 2 Kinetic properties of the winged bean kinase

The protein kinase assays were performed for 30 s at different concentrations of [γ-^{32}P]ATP and MLC-peptide with 1 μg of the enzyme in a total volume of 50 μl, and ^{32}P incorporation was determined by the gel assay method. The kinetic parameters were obtained from Lineweaver–Burk double reciprocal plots.

Substrate	K_m	$V_{max.}$ (nmol/min mg^{-1})
ATP	16.4 μM	4.14
MLC-peptide	14.3 μM	4.34
Syntide 2	7.8 μM	6.67
Histone H1	0.07 mg/ml	1.82

found to be good substrates and among the three, syntide 2 appears to be best exogenous substrate (Table 2).

The enzyme undergoes autophosphorylation and phosphoserine is the only phospho-amino acid identified in the acid hydrolysate of the autophosphorylated enzyme (Figure 2a). Two-dimensional PAGE analysis shows that both the 61 and 58 kDa polypeptides become autophosphorylated and have similar pI values (Figure 2b). The time course of the reaction, as presented in Figure 2(c), clearly shows that autophosphorylation reaches an apparent saturation in about 90 min and at this stage, an incorporation of 1.04 mol of P_i/mol of enzyme (based on an M_r of 60000), is estimated. A more or less constant relative specific activity of the autophosphorylating enzyme, yields a slope of 1.1 for the van't Hoff plot [32] over a wide range of enzyme concentration (Figure 2d) attesting to the intramolecular mechanism of autophosphorylation.

Ca^{2+}-dependence of the enzyme for substrate phosphorylation but not for autophosphorylation

EGTA brings about a total loss of substrate phosphorylating activity of the purified enzyme, which can be restored by the addition of Ca^{2+} (Figure 3a, lower panel). This establishes the Ca^{2+}-dependence of the enzyme for substrate phosphorylation, which remains unaltered even after partial autophosphorylation, as depicted in the same Figure by the abolition of substrate phosphorylation by a second addition of EGTA at 10 min.

Autophosphorylation of the enzyme, unlike its substrate phosphorylation, is not effected by Ca^{2+} or EGTA (Figure 3a, upper panel), revealing the Ca^{2+}-independence for the reaction.

Like EGTA, fluphenazine drastically inhibits substrate phosphorylation catalysed by the winged bean enzyme. As shown in Figure 3(b), more than 50% inhibition of MLC-peptide phosphorylation is observed at 50 μM fluphenazine whereas even at 1 mM concentration of the inhibitor, autophosphorylation remains unchanged. Although exogenous CaM by itself does not enhance substrate phosphorylation catalysed by the purified enzyme, it partially reverses the inhibition of substrate phosphorylation by fluphenazine (results not shown), indicating the possible participation of a Ca^{2+}-binding protein present either as a separate entity in the preparation or as an integral domain of the purified enzyme. Figure 3(c) shows that both the 61 and 58 kDa polypeptide components of the purified enzyme bind $^{45}Ca^{2+}$, after SDS denaturation, SDS/PAGE, transfer and renaturation. Bovine CaM is used as the positive control.

These findings strongly suggest the presence of an integrated Ca^{2+}-binding domain in the winged bean enzyme as in the recently classified CDPKs [12].

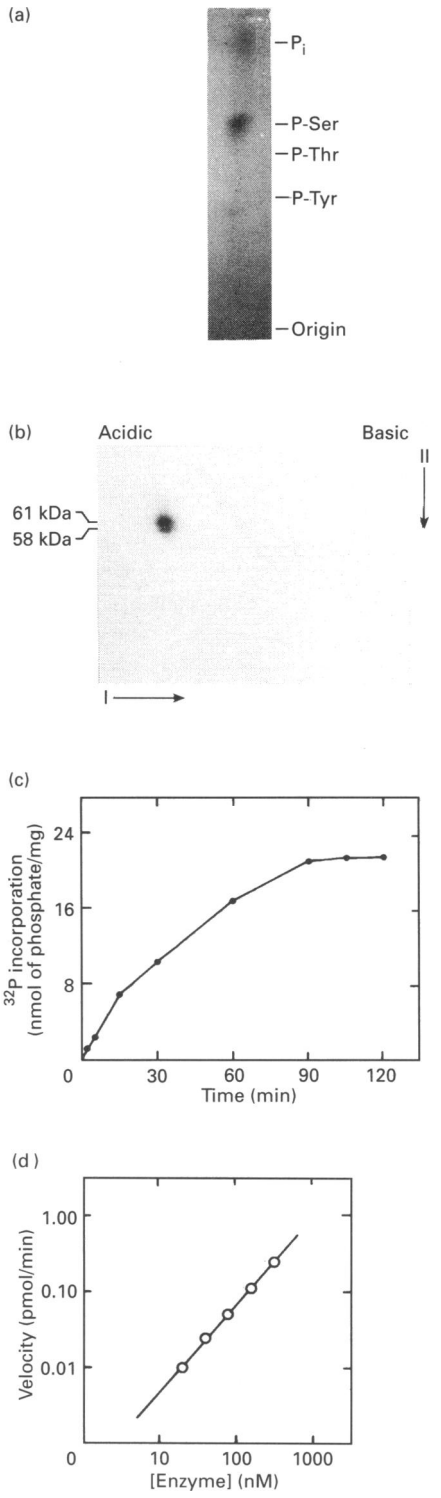

Figure 2 Autophosphorylation of the winged bean protein kinase

The purified enzyme was autophosphorylated by incubating with [γ-^{32}P]ATP in the absence of peptide substrates. (**a**) Identification of phospho-amino acids. The autophosphorylated enzyme was hydrolysed in 6 M HCl at 110 °C for 4 h and the hydrolysate was analysed by electrophoresis on thin layer cellulose sheets at 450 V for 90 min using a pyridine/acetic acid/H_2O (5:50:945) buffer system, followed by autoradiography [34]. (**b**) Two-dimensional PAGE of the autophosphorylated enzyme was carried out [33], followed by autoradiography. Direction I, non-equilibrium pH-gradient (pH 3.5–10) gel electrophoresis for 1000 V h. Direction II, SDS/PAGE (12% acrylamide). (**c**) Time course of autophosphorylation. Rate of ^{32}P incorporation into the enzyme was measured by the P81-binding method. (**d**) van't Hoff plot [32] of log velocity versus log enzyme concentration.

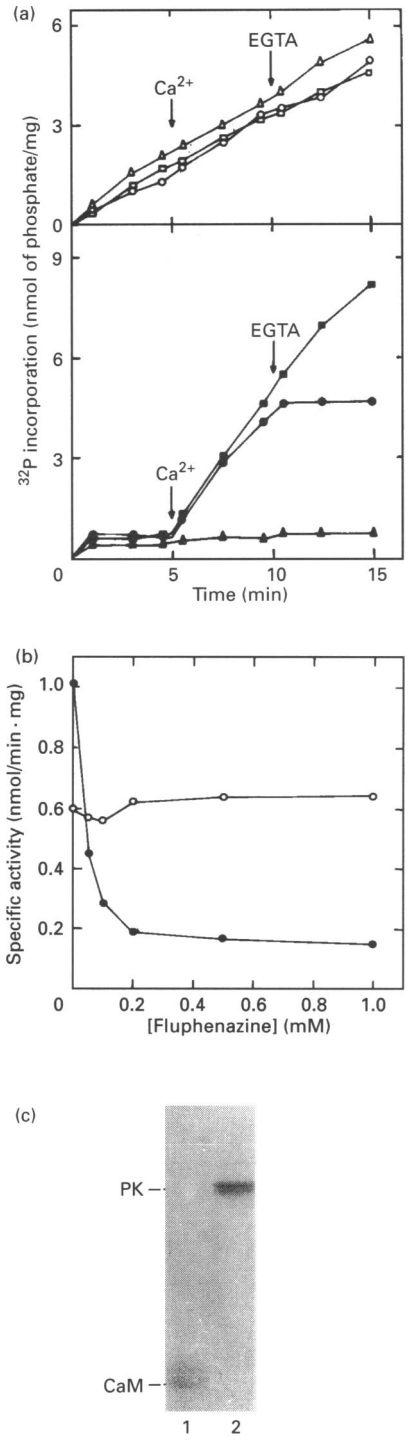

Figure 3 Effect of Ca^{2+} and fluphenazine on the winged bean protein kinase

(**a**) Effect of Ca^{2+}. The purified winged bean protein kinase was incubated in three sets (in duplicate) in standard reaction mixtures containing 50 μM MLC-peptide and 0.2 mM EGTA in the following manner: Set 1, at 5 min of incubation Ca^{2+} was added (final concn., 0.5 mM), followed by EGTA at 10 min (final concn., 1.4 mM); Set 2, Ca^{2+} was added as in Set 1 at 5 min, but only an equal volume of buffer was added at 10 min; Set 3, equal volumes of buffer were added at 5 and 10 min. Portions of the reaction mixtures were withdrawn at different time intervals, mixed with SDS sample buffer [24] and the [^{32}P]phosphorylated products were analysed by the gel assay method as described in the Materials and methods section. The solid symbols (lower panel) represent the substrate phosphorylation and the open symbols (upper panel) indicate the autophosphorylation. (●,○), Set 1; (■, □), Set 2; (▲, △), Set 3. (**b**) Effect of fluphenazine. Phosphorylation reactions were carried out in the presence of 0.5 mM $CaCl_2$ and varying concentrations of fluphenazine for 10 min and the [^{32}P]phosphorylated products were analysed by the gel assay method. ○, Autophosphorylation; ●, MLC-peptide phosphorylation. (**c**) Binding of $^{45}Ca^{2+}$. The purified winged bean protein kinase and bovine brain CaM were subjected to SDS/PAGE (12% acrylamide) and transferred to nitrocellulose paper, and Ca^{2+}-binding on the nitrocellulose blot was performed with $^{45}CaCl_2$ according to Maruyama et al. [35]. Lanes: 1, CaM; 2, winged bean protein kinase (PK).

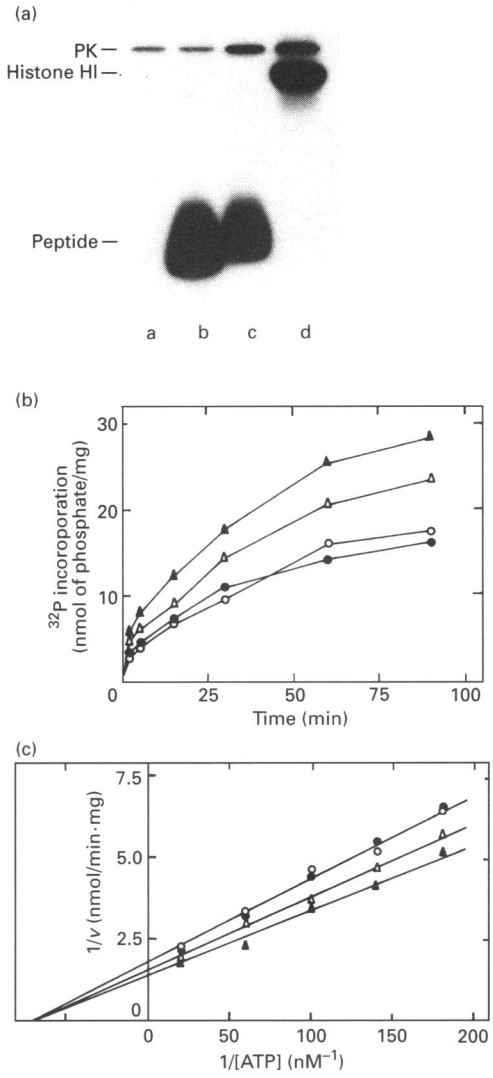

Figure 4 Effect of protein/peptide substrates on the autophosphorylation of the winged bean kinase

The purified protein kinase (PK) was incubated in standard reaction mixtures in the presence or absence of exogenous protein/peptide substrates. The [^{32}P]phosphorylated products were analysed by SDS/PAGE (20%) and the gel assay method. (**a**) Autoradiography of the [^{32}P]phosphorylated products. The reactions were carried out for 10 min. Lanes: a, no protein/peptide substrates; b, 50 μM syntide 2; c, 50 μM MLC-peptide; d, histone H1 (0.1 mg/ml). (**b**) Time course of autophosphorylation in the presence or absence of protein/peptide substrates. (**c**) Lineweaver–Burk plot of autophosphorylation in the presence or absence of protein/peptide substrate. The kinetic measurements were carried out as described in Table 2. Symbols for (**b**) and (**c**): ○, no protein/peptide substrate; ●, 50 μM syntide 2; △, 50 μM MLC-peptide; ▲, histone H1 (0.1 mg/ml).

Effect of substrates on the rate as well as the extent of autophosphorylation by winged bean CDPK (WbCDPK)

Comparison of autophosphorylation by WbCDPK (Figure 4a) in the absence (lane a) and the presence of the three known

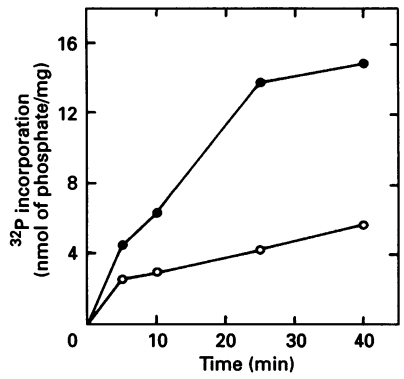

Figure 5 Down-regulation of the protein kinase by autophosphorylation

The protein kinase was preincubated in a reaction mixture without the MLC-peptide but containing 50 μM ATP (non-radioactive) for 2 h to achieve maximum autophosphorylation. MLC-peptide and [γ-^{32}P]ATP were then added and the final concentration of ATP was adjusted to 50 μM with the specific activity of 2000 c.p.m./pmol. Further incubation was carried out and suitable portions were taken at different times into SDS sample buffer. ^{32}P incorporation into the peptide was determined by the gel assay method. For the control, ATP was omitted at the time of preincubation. The Figure shows MLC-peptide phosphorylation by phosphorylated protein kinase (○) and by control enzyme (●).

exogenous substrates shows that syntide 2 (lane b) does not have any effect on it while MLC-peptide (lane c) and histone H1 (lane d) enhance the level of autophosphorylation by about 60% and 100% respectively. Since both histone H1 and MLC-peptide are basic, the possible effect of polyamines (spermine, spermidine and putrescine) on the autophosphorylation of WbCDPK has been tested and found to be ineffective (results not shown). Hence, the enhancements by histone H1 and MLC-peptide are considered as specific effects.

The effect of the substrates on the rate as well as the extent of autophosphorylation is represented by the time course of the reaction (Figure 4b). Maximum incorporation of P_i (mol/mol of enzyme) has been estimated to be about 1 (for control and in the presence of syntide 2), 1.4 (in the presence of MLC-peptide) and 1.7 (in the presence of histone H1). Figure 4(c) presents the double reciprocal plot ($1/v$ versus $1/ATP$) for autophosphorylation revealing that the enhancement by MLC-peptide and histone H1, are due to the increase in $V_{max.}$ rather than the decrease of K_m for ATP.

Down-regulation of substrate phosphorylation by phosphorylated enzyme

The role of autophosphorylation has been studied by following the time course of substrate phosphorylation, catalysed by autophosphorylated protein kinase. As presented in Figure 5, the MLC-peptide phosphorylation is greatly reduced by prior autophosphorylation.

DISCUSSION

The results of the present studies have shown that the purified winged bean protein kinase is Ca^{2+}-dependent for its substrate phosphorylation activity, but it does not require CaM or phospholipids. However, substantial inhibition by fluphenazine and the reversal by a heterologous CaM, albeit only partially, taken together with results of Ca^{2+}-binding on blot after SDS/PAGE and subsequent renaturation, are clear indications of the presence of a CaM-like Ca^{2+}-binding domain which is involved in the regulation of enzymic activity. These findings are also compelling evidence for the classification of the winged bean enzyme as a new member of the CDPK family, represented by the protein kinases from soybean [17], groundnut [20], *A. thaliana* [19], *P. tetraurelia* [16], etc., the presence of the CaM-like domain being the diagnostic structural feature.

CDPKs, including soluble and membrane-associated forms, have been isolated from diverse plant sources [13] and in some cases even from the same tissue [18]. Some of these are activated by phospholipids as in the case of membrane-bound oat root CDPK [18] and the *Arabidopsis* CDPK [19]. In the present studies, a membrane-bound isoform of WbCDPK has not been detected by immunoblot analysis (Figure 1c). Differences among CDPKs are expected since Ca^{2+}-mediated regulation is the only common link between them. Therefore, the present finding of phosphoserine in the autophosphorylated WbCDPK is not surprising, although phosphoserine and phosphothreonine have been reported for some other CDPKs [17].

The commonality as well as the diversity among the CDPKs makes sense when examined in the light of the possible evolution of CDPKs by fusion of pre-existing genes, as proposed by Harper et al. [11]. It is conceivable that the fusion of catalytic domains from divergent protein kinases with a pre-existing, conserved CaM-like sequence would give rise to different CDPKs having more or less similar Ca^{2+}-binding properties and thus accounting for the present as well as the previous observations.

In spite of the finding of two polypeptide chains of 61 and 58 kDa by SDS/PAGE, the purified WbCDPK is adjudged to be a single, monomeric, catalytic entity, with an approximate average molecular mass of 60 kDa, since both the polypeptides undergo autophosphorylation (Figure 2b) following an intramolecular mechanism (Figure 2d). The latter is particularly significant since it shows that both of them are catalytically active and their phosphorylation is not catalysed by a contaminating kinase. This conclusion is also consistent with the direct Ca^{2+}-binding by both the polypeptides (Figure 3c). Further evidence indicating close structural homology between the two is provided by the apparently identical pI values as well as the results of immunoblot analysis, showing their cross-reactivity with the two antisera raised against the individual polypeptides. Since a single, larger polypeptide (62 kDa) is detectable in the freshly prepared tissue extracts instead of the doublet of 61 and 58 kDa (Figure 1c), the likelihood of the doublet being formed from the 62 kDa protein by proteolysis is strongly indicated. This is further supported by the requirement of protease inhibitors in the homogenization buffer to obtain a stable preparation which does not undergo rapid loss of activity and degradation on storage. Protease interference in protein purification is a matter of common experience, and the occurrence of such doublets, attributable to proteolysis have been reported for many enzymes, including protein kinases from soybean [17], rat pancreas [8] and bovine brain [36].

The Ca^{2+}-independent autophosphorylation of the WbCDPK is an unique property, shared by only one other enzyme, i.e. the casein kinase-like CDPK from *Paramecium* [16], and merits further consideration in the light of the possible regulatory mechanism of CDPK, as postulated by Roberts [13]. Although it has not been fully worked out, the importance of the junction sequence, particularly the putative auto-inhibitory domain contained in this sequence, has been realized [12–14]. In analogy to the known mechanism of animal CaM-kinase II, it is suggested that at low intracellular levels of Ca^{2+} (0.1 μM or less), the enzyme is inactivated by the interaction of the auto-inhibitory domain with a sequence at or near the catalytic site, thus hindering the binding of either both or one of the two substrates i.e. the protein substrate and the nucleotide. At higher Ca^{2+}

concentrations (1.0 μM or more), the Ca^{2+}-ligated EF-hand domain could bind with a positively charged amphipathic α-helical structure adjacent to the auto-inhibitory region and thus activate the enzyme [6]. This mechanism accounts for the participation of both Ca^{2+} and the Ca^{2+}-binding domain during substrate phosphorylation. However, the unusual Ca^{2+} autonomy observed in the case of autophosphorylation of WbCDPK can be explained on the basis of blocking of the active site only partially by the auto-inhibitory domain affecting only the peptide-binding site. Therefore, ATP can bind the enzyme independently of the intracellular level of Ca^{2+}, whereas the protein substrate needs the unblocking of the active site at higher levels of Ca^{2+}.

Our present finding of enhancement of WbCDPK autophosphorylation by histone H1 and MLC-peptide, two of its substrates, is quite convincing (Figure 4). While the significance of this finding is not clear, at the same time we are not aware of any other protein kinase the autophosphorylation of which is regulated in such a way. Nevertheless, we have considered the possibility of a conformational change as a consequence of binding histone H1 or MLC-peptide but not syntide 2, and this may lead to the exposure of different phosphorylation site(s). Such a possibility can be properly assessed after the phosphorylation sites are identified.

The present finding that autophosphorylation does not alter the Ca^{2+}-dependence of WbCDPK for substrate phosphorylation (Figure 3a) indicates that there is no change in the masking of the peptide-binding site of the auto-inhibited enzyme. This may also mean that the site of phosphorylation does not favour the unmasking of the substrate-binding site of the enzyme, unlike in the case of autophosphorylated multifunctional CaM kinase II [6].

The down-regulation of substrate phosphorylation as a result of autophosphorylation (Figure 5) may also be directly related to the site of phosphorylation. It is possible to account for the down-regulation on the basis of either an impairment of the binding of the Ca^{2+}-ligated EF-hand domain to the putative CaM-binding site in the junction sequence or an alteration in the peptide-binding site of the catalytic centre of the enzyme as a result of autophosphorylation. Since nothing much is known about the site of phosphorylation it is not possible to choose between these alternatives. However, the down-regulation by autophosphorylation may be part of a negative feedback circuit, involving a phosphatase, and such a mechanism could be physiologically significant.

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