Intramolecular Binding Contributes to the Activation of CDPK, a Protein Kinase with a Calmodulin-Like Domain†

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ABSTRACT: The activity of calmodulin-like domain protein kinase (CDPK) is regulated by the direct binding of Ca^{2+}. Unmodified soybean CDPKα and a chimeric enzyme in which the calmodulin-like domain (CLD) was replaced by VU-1 calmodulin had similar values of $V_{max_{app}}$ (3.19, 3.46, and 3.60, 3.93 μmol/min/mg, respectively), and each was activated 30−70-fold by Ca^{2+}. To determine if activation results from the binding of the CLD to the autoinhibitory (junction) domain of CDPKα in a manner analogous to the activation of calmodulin-dependent enzymes by calmodulin, recombinant CLD and truncation mutants of CDPKα were expressed in bacteria and highly purified. In blot overlays, biotinylated CLD bound to mutants containing residues 312−328 of the junction domain. In an electrophoretic mobility shift assay CLD bound synthetic peptides containing residues 318−332 in a calcium-dependent manner, providing direct evidence for binding of CLD to a site in the junction domain. Mutants of CDPKα from which all or part of the CLD had been deleted were constitutively inactive. Addition of 20 μM CLD to these mutants in the presence, but not the absence, of calcium stimulated their activities, but to various degrees. His_6-CDPKα(1−328), which contained none of the CLD, was activated only 5-fold, but the activity of His_6-CDPKα(1−398), which retained nearly half of the CLD in its sequence, was stimulated 64-fold. The latter activity approached that of unmodified CDPKα and was half maximal at a CLD concentration of 7 μM. Our results suggest that binding of CLD to the junction domain contributes to, but is not sufficient for activation. Although calmodulin supported full activity of the chimeric enzyme, its addition to His_6-CDPKα(1−398) resulted in activity that was only 6% of that of the unmodified enzyme and which was half-maximal at 20 μM *Arabidopsis* calmodulin. These results support the conclusion that simple binding of the calmodulin-like domain to the junction domain is not sufficient for activation.

It is well established that calcium plays a role as a second messenger in diverse physiological processes in plants (Bush, 1993; Gilroy et al., 1993; Hepler & Wayne, 1985; Poovaiah & Reddy, 1993). The principal targets of calcium signals are calcium-modulated proteins, and the most well-studied calcium-modulated proteins in plants are calmodulin and calcium-dependent, calmodulin-independent protein kinase (CDPK)[1] (Roberts & Harmon, 1992). Calmodulin has no intrinsic enzymatic activity, but when complexed with Ca^{2+} it binds to and modulates the activities of various enzymes. In contrast, calmodulin-like domain protein kinase (calcium-dependent protein kinase, or CDPK) is activated by the direct binding of Ca^{2+} to its regulatory domain that is similar in primary structure to calmodulin (Harmon et al., 1987; Harper et al., 1991; Putnam-Evans et al., 1990). This unusual structural feature places CDPK in a new class of calcium-regulated protein kinases.

CDPKs contain four domains (listed in order from the amino terminus to the carboxyl terminus): an amino-terminal domain that varies in length and sequence among CDPKs and has no similarity to other proteins; a protein kinase catalytic domain most closely related in sequence to the catalytic domains of Ca^{2+}/calmodulin-dependent protein kinases; a junction domain that functions in autoinhibition; and a calmodulin-like domain. The calmodulin-like domain (CLD) of CDPKs is 30%−40% identical to calmodulin from seed plants or mammals, and it confers calcium sensitivity to the enzyme (Harmon et al., 1994; Harper et al., 1994; Zhao et al., 1994). As is true for calmodulin, all four EF-hands of *Plasmodium* CDPK bind Ca^{2+} (Zhao et al., 1994). Both calmodulin and soybean CDPK bind phenothiazine and naphthalenesulfonamide compounds in a calcium-dependent manner (Harmon et al., 1987; Putnam-Evans, 1986; Putnam-Evans et al., 1990). These observations suggest that CLD and calmodulin have some functional similarities.

Previous studies showed that the junction domain is responsible for autoinhibition of the enzyme probably via a pseudosubstrate mechanism (Harmon et al., 1994; Harper et al., 1994). The pseudosubstrate is a sequence that has

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[1] Abbreviations: CDPK, calmodulin-like domain protein kinase; CDPKα, protein encoded by soybean cDNA SK5; CLD, calmodulin-like domain; CaMPKII, calcium/calmodulin-dependent protein kinase type II; PMSF, phenylmethylsulfonyl fluoride; TPCK, tosyl-L-phenylalanine chloromethyl ketone, MLCK, myosin light chain kinase; CaM, calmodulin; ACaM-2, *Arabidopsis* calmodulin-2; VU-1 calmodulin, calmodulin encoded by a synthetic gene (Roberts et al., 1985); PCR, polymerase chain reaction.

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features of phosphorylation sites recognized by the enzyme but which is missing a phosphorylatable residue. This sequence interacts with the active site and blocks entry of substrate (Kemp & Pearson, 1991; Soderling, 1990). Removal of the junction and calmodulin-like domains from CDPK results in a constitutively active enzyme, while removal of the calmodulin-like domain alone results in an enzyme that is inactive (Harmon et al., 1994; Harper et al., 1994). A study with synthetic peptides showed that a peptide corresponding to residues 310−332 of the junction domain of soybean CDPKα competitively inhibited activity with respect to a peptide substrate (Harmon et al., 1995). These observations support the hypothesis that CDPKs are autoinhibited in the absence of calcium by a pseudosubstrate mechanism.

A well-documented conformational change occurs in calmodulin upon binding Ca^{2+} that allows the Ca^{2+}/CaM complex to bind to target enzymes and modulate their activities (Ikura et al., 1992). If a similar conformational change occurs in CLD in the presence of calcium, such a change could bring about activation of CDPK. Since CLD is covalently attached and is adjacent to the junction domain of CDPK, it is possible that a conformational change could be transmitted through the peptide chain to the junction domain, and activate the enzyme (the conduit model). Alternatively, the conformational change could lead to binding of CLD to the junction domain (the binding model) in a mechanism similar to the activation of CaMKII and MLCK by calmodulin (Soderling, 1990; Somlyo & Somlyo, 1994). It is also possible that a combination of the two mechanisms occurs.

In this work, we used a molecular genetic approach to investigate the nature of the activation of CDPK by Ca^{2+}. A chimeric CDPK in which calmodulin was substituted for the calmodulin-like domain of CDPKα was constructed and shown to be fully active and regulated by Ca^{2+}. To determine whether activation of the CDPKα is achieved by binding between the junction domain and calmodulin-like domain, the calmodulin-like domain (CLD) was individually expressed and tested for binding and activation of a series of mutant CDPKαs in which all or part of the CLD had been deleted. Our results show that binding of the CLD to the junction domain contributes to activation but indicate that binding is not sufficient for complete activation.

EXPERIMENTAL PROCEDURES

Materials. Syntide-2 (Hashimoto & Soderling, 1987) and CDPKα peptides (Harmon et al., 1994) were synthesized by the Protein Chemistry Core Laboratory at the University of Florida. Glutathione agarose and iminodiacetic acid-Sepharose were purchased from Sigma. pGEX-KG (Guan & Dixon, 1991) was a gift from Dr. E. Schaller (University of Wisconsin), as were pVUCH1 (Roberts et al., 1985) from Dr. D. Roberts (University of Tennessee), ACaM-2 from Dr. R. Zielinski (University of Illinois), CaMPKII from Dr. T. Soderling (Vollum Institute, Oregon Health Sciences Center, Portland, OR), and pETh-3b from Dr. D. McCarty (University of Florida). *Escherichia coli*, PR 745, was from Dr. D. Randall (University of Missouri).

Plasmid Construction and Site-Directed Deletion Mutagenesis. Plasmids pHis1530 and pAY, each encoding full-length CDPKα, were described previously (Harmon et al., 1994). Deletion mutagenesis for constructing $pHis_6$-CDPKα(1−328), $pHis_6$-CDPKα(1−333), $pHis_6$-CDPKα(1−339), $pHis_6$-CDPKα(329−508), and pGEX-KG/CDPKα(1−312) was performed by employing expression-cassette PCR (Macferrin et al., 1990) and pAY as a template. The numbers in parentheses indicate amino acid residues of CDPKα. $pHis_6$-CDPKα(1−398) was made by exploiting restriction sites, and the resulting encoded polypeptide contained four extra, vector-encoded amino acid residues (LIHR) at the carboxyl terminus. For construction of $pHis_6$-CDPKα(1−328)-VUCH1, which encodes a CDPKα-calmodulin chimera, a cDNA insert encoding CDPKα(1−328) was amplified by PCR and ligated into an intermediate vector, pUC19. pVUCH1 was digested with *Bcl*I and *Bam*HI and ligated to the intermediate vector. The resulting plasmid was cut with *Nde*I and *Bam*HI and cloned into the expression vector, pETh-3b. Other expression vectors used were pET-15b (Novagen) and pGEX-KG. Sequences of all constructs were verified (Sequenase version 2.0, Amersham Life Science).

Expression and Purification of Full-Length and Mutants of CDPKα. *E. coli* hosts for the expression of pETh-3b- and pET-15b-derived vectors were BL21(DE3) or BL21-(DE3)pLys S, and the host for pGEX-KG-derived vectors was PR 745. Protease inhibitors (1 mM PMSF, 10 μg of leupeptin/mL, and 20 μg of aprotinin/mL) were added to all of the cell resuspension buffers. Extraction and purification steps were performed at 4 °C. Cells expressing His_6-fusion proteins were resuspended in binding buffer (20 mM Tris, 0.5 M NaCl, 5 mM imidazole, pH 8.0) and sonicated, and cellular debris was pelleted by centrifugation. The supernatant was made 1% (v/v) in Triton X-100 and filtered through a 0.2 μm filter. The filtrate was loaded onto a nickel chelation column prepared from iminodiacetic acid-Sepharose and connected to an FPLC (Pharmacia). The column was washed with 10 column volumes of wash buffer (20 mM Tris, 0.5 M NaCl, 60 mM imidazole, pH 8.0) and eluted with elution buffer (20 mM Tris, 0.5 M NaCl, 400 mM imidazole, pH 8.0). Fractions containing expressed proteins were pooled and dialyzed overnight with three 4 L changes of Mono Q equilibration buffer (20 mM Tris, pH 8.0, 2.5 mM EDTA). Dialyzed samples were loaded onto a Mono Q, HR 5/5 column (Pharmacia) and eluted with a gradient of 0−0.5 M NaCl in equilibration buffer. Fractions of the highest purity as determined by SDS−PAGE were pooled and concentrated by ultrafiltration (Centricon 10, Amicon). Concentrated proteins were washed with 20 mM Tris, pH 8.0, and 0.1 mM DTT while in the concentration device. Glycerol was added to the final concentration of 10% or 50% (v/v), and the enzyme was stored at −70 or −20 °C.

Cells expressing pGEX-KG/CDPKα(1−312) were treated as above except that the cell resuspension buffer (buffer A) was 50 mM Tris, pH 8.0, and 150 mM NaCl. The extract was loaded onto a glutathione-agarose column equilibrated in buffer A. After extensive washes with buffer A, bound proteins were eluted with 50 mM Tris and 10 mM reduced glutathione, pH 7.2. Fractions containing kinase activity were pooled and chromatographed on Blue-Sepharose as previously described (Putnam-Evans et al., 1990).

Recombinant calmodulin-like domain (CLD) was purified by a modification of methods used for the purification of calmodulin (Anderson, 1983; Roberts et al., 1985). Cells expressing CLD were resuspended in lysis buffer (50 mM Tris, pH 7.2, 0.5 M NaCl, 2 mM EDTA, 1 mM DTT) and

sonicated. Clarified cell extracts were boiled for 5 min, and denatured proteins were pelleted by centrifugation. The supernatant was made 5 mM in $CaCl_2$ and was loaded on phenyl-Sepharose equilibrated with 20 mM Tris, 1 mM $CaCl_2$, pH 7.2. After extensive washing with 20 mM Tris, pH 7.2, and 0.5 mM $CaCl_2$, CLD was eluted with 20 mM Tris and 2.5 mM EDTA, pH 7.2. Pooled fractions from phenyl-Sepharose were applied to Mono Q HR 5/5 equilibrated with 20 mM Tris, 2.5 mM EDTA, pH 7.2. Proteins were eluted with a 0−0.5 M NaCl gradient in the same buffer. Fractions containing CLD were dialyzed extensively against 5 mM NH_4HCO_3 and then against 20 mM Tris, pH 7.2, and 0.1 mM DTT.

Biotinylation of Calmodulin-Like Domain and Overlay. CLD was biotinylated as described by Billingsley et al. (1985) with Biotin-X-NHS (Calbiochem) in the presence of 1 mM $CaCl_2$. For the overlay assay, proteins were separated by electrophoresis in SDS−polyacrylamide gels in the presence of 1 mM EGTA and electroblotted to nitrocellulose. Blots were blocked in 5% (w/v) nonfat dry milk, 50 mM Tris, pH 7.5, and 300 mM NaCl for 1 h at room temperature with constant agitation and washed in TBS (50 mM Tris, pH 7.5, 300 mM NaCl) for 10 min. Blots were incubated in TBS containing 1% (w/v) gelatin with biotinylated CLD (5 μg/mL) for 2 h in the presence of 1 mM $CaCl_2$ and then washed twice with TBS plus 1 mM $CaCl_2$. Biotinylated CLD was detected with ExtrAvidin-alkaline phosphatase conjugate (Sigma) at a 1:1000 dilution. Control experiments contained 5 mM EGTA in place of $CaCl_2$.

Protein Kinase Assays. Activity assays were performed as described by Harmon et al. (1994) with the following modification. The assay buffer contained 50 mM HEPES, pH 7.2, 10 mM $MgCl_2$, 1 mM EGTA, 0.4 mg of BSA/mL, with or without 1.1 mM $CaCl_2$. Truncated CDPKαs were assayed in a volume of 25 μL with 200 μM syntide-2 (peptide substrate), 20 nM recombinant enzyme, and the indicated concentrations of CLD. BSA was added in place of CLD to keep the total protein concentration constant in all tubes. The dependence of the activity of His_6-CDPKα-(1−398) on the concentration of CLD was done as above except with 100 μM syntide-2, 1 mM DTT, and 1 mg of BSA/mL. The activity of recombinant calcium/calmodulin-dependent protein kinase II was determined as described in Harmon et al. (1994) with 2 or 20 μM CLD and with ACaM-2 as a control.

To facilitate comparison of activities of proteins of different sizes, activities are reported on a molar basis (μmol min^{-1} $\mu mol^{-1} = min^{-1}$). Moles of enzymes were calculated from molecular weights predicted from cDNA sequences: His_6-CDPKα, 59 339; His_6-CDPKα(1−398), 47 149; His_6-CDPKα(1−339), 40 403; His_6-CDPKα(1−333); 39 788; His_6-CDPKα(1−328), 39 232; GST-CDPKα(1−312), 63 160; CLD, 21 124; ACaM-2, 16 689.

Tryptic Digestion. CDPKα and truncation mutants (0.2 mg/mL) were digested with 10 μg of trypsin/mL (TPCK-treated, Worthington) in buffer containing 1 mM EGTA, 30 mM NaCl, and 30 mM Tris-HCl (pH 7.5) at 25 °C. At various times an aliquot was removed and added to 100 μg of soybean trypsin inhibitor/mL to terminate digestion. Activity was determined as described above.

Electrophoresis. SDS−PAGE was conducted according to the method of Laemmli (1970). Glycerol−PAGE was performed as described in Persechini et al. (1986) with the following modifications. Samples were prepared by combining calmodulin-like domain (100 pmol) with 100 or 1000 pmol of indicated CDPKα peptides in the presence of 1 mM $CaCl_2$ or 2 mM EGTA. Samples were electrophoresed at 300 V and 10 °C for 28−48 h in 1.5 mm polyacrylamide slabs (16 × 18 cm) containing 10% (w/v) acrylamide, 0.5% (w/v) bisacrylamide, 40% (w/v) glycerol, 20 mM Tris, 23 mM glycine, pH 8.6, and with either 1 mM $CaCl_2$ or 2 mM EGTA. Pre-electrophoresis was performed at 400 V and 10 °C for 1.5 h. The reservoir buffers contained 20 mM Tris, pH 8.6, 23 mM glycine, and either 1 mM $CaCl_2$ or 2 mM EGTA. The reservoir buffers were replaced several times during electrophoresis. Urea/glycerol gel electrophoresis was carried out as above except 4 M urea was added to the polyacrylamide gel and samples.

Other Procedures. The concentration of syntide-2 was determined from amino acid composition analysis done in the Protein Sequencing Core Facility, University of Florida. The concentration of recombinant proteins were measured by the Bio-Rad dye-binding assay, based on the method of Bradford (1976). Concentrations of CLD and *Arabidopsis* CaM-2 were estimated with the method of Lowry et al. (1951). Digital images of polyacrylamide gels and nitrocellulose blots were obtained as previously described (Harmon et al., 1994).

RESULTS

CDPKα-Calmodulin Chimera is Similar to CDPKα in Activity. To assess whether calmodulin is able to function in place of CDPKα's CLD, a chimeric enzyme was made in which CLD was replaced by a synthetic CaM, VU-1. The sequence of VU-1 is a conservative hybrid between plant and vertebrate CaM, and the protein is functionally comparable to CaM (Roberts et al., 1985). The chimeric enzyme contained the first 328 amino acids of CDPKα (the amino-terminal, catalytic, and junction domains), but CLD (the carboxyl-terminal 180 residues of CDPKα, $_{329}$AERL...GY-FK_{508}) was replaced by the complete VU-1 sequence ($_1$ADQL...$MMAK_{148}$). The number of residues separating the junction domain and the first EF hand was conserved between the chimeric enzyme and CDPKα (Figure 1).

Both CDPKα and the chimera were expressed as His_6-tagged fusion proteins and purified by metal chelation chromatography. Since a high degree of purification was essential in order to accurately assess the effect of the substituted sequence, the proteins were purified further on Mono Q HR 5/5 (A and B in Figure 2). Both proteins were eluted at 200 mM NaCl.

The activities of CDPKα and the chimera were stimulated 30−70-fold by calcium, and both enzymes had similar values of apparent V_{max} (Table 1). The apparent K_M for syntide-2 was slightly higher for the chimera than for CDPKα (Table 1). These results show that calmodulin can mimic the properties of CLD in transducing the calcium signal to activate fully the catalytic activity of CDPK. While these results suggest that CLD and VU-1 may use a mechanism involving Ca^{2+}-dependent conformational changes to activate the protein kinase, they do not reveal what the mechanism is. We undertook the following work to ask if activation results from the binding of the CLD to the junction domain of CDPKα in a manner analogous to the activation of calmodulin-dependent enzymes by calmodulin.

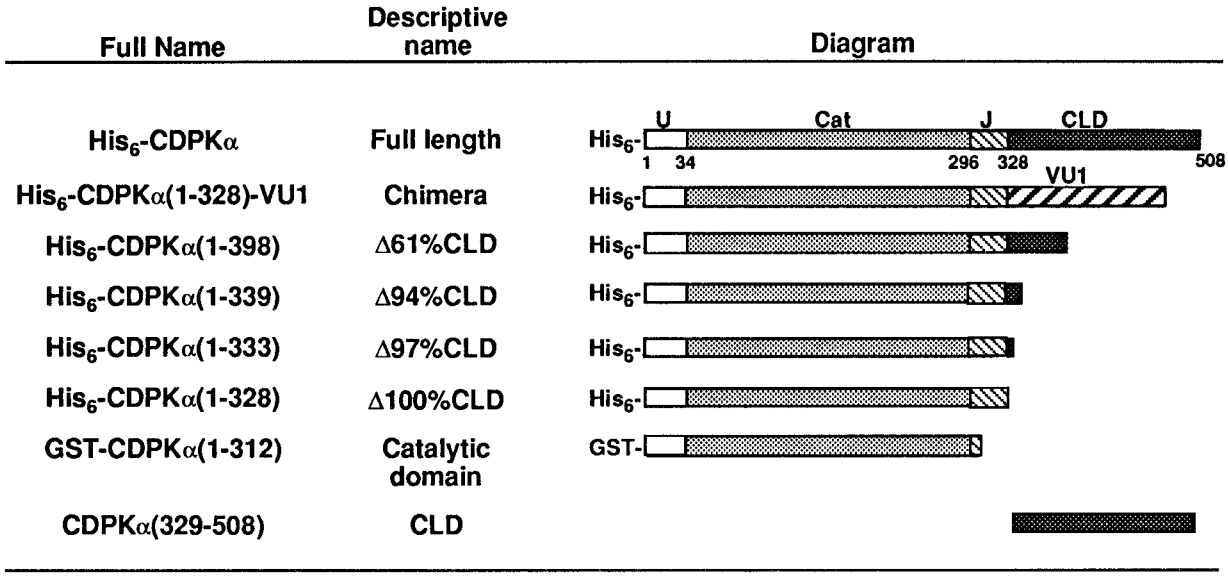

FIGURE 1: Structural features of recombinant proteins used in this study. Unmodified CDPKα was expressed as fusion protein with an affinity tag containing six histidine residues (His_6) at the amino terminus. The domains of CDPKα are labeled as follows: amino-terminal domain of undefined function (U), catalytic domain (Cat), junction domain (J), calmodulin-like domain (CLD). Residue numbers marking the boundaries of domains are indicated below the molecular diagram. The chimeric enzyme has the same structure as CDPKα except that synthetic calmodulin (VU-1) replaces CLD. The ΔCLD mutants were produced by deletion of all or part of the CLD. The constitutively active mutant GST-CDPKα(1−312), which contains the amino-terminal and catalytic domains plus a few residues of the junction domain, was expressed as a fusion protein with glutathione *S*-transferase (GST) at the amino terminus. CLD (residues 329−508 of CDPKα) was not expressed as a fusion protein.

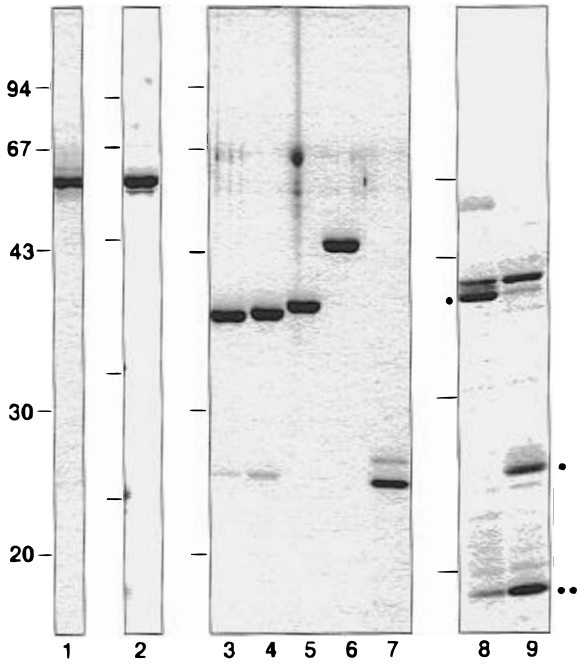

FIGURE 2: Purity of recombinant proteins. Purified recombinant proteins (2 μg) were analyzed by electrophoresis in 12% polyacrylamide gel in the presence of SDS, which was stained with Coomassie Blue. Lane 1, His_6-CDPKα; 2, His_6-CDPKα(1−328)-VU-1; 3, His_6-CDPKα(1−328); 4, His_6-CDPKα(1−333); 5, His_6-CDPKα(1−339); 6, His_6-CDPKα(1−398); 7, CLD; 8, GST-CDPKα(1−312); 9, GST-CDPKα(1−312) treated with thrombin for 50 min. The dot to the left of lane 8 indicates the position of GST-CDPKα(1−312). The positions of CDPKα(1−312) and GST in lane 9 are indicated by single and double dots, respectively. The positions of molecular mass markers are indicated by the lines to the left of each panel. From top to bottom they are phosphorylase b, 94 kDa; BSA, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; and soybean trypsin inhibitor, 20 kDa.

Expression, Purification, and Properties of Truncation Mutants. If binding of CLD to the junction domain is the major event that activates the enzyme, then CLD should not have to be covalently attached to the rest of the enzyme in order to accomplish activation. In preparation for testing this prediction, several truncation mutants (Figure 1) were expressed, purified, and characterized.

Table 1: Kinetic Parameters of CDPKα and the Chimeric Enzyme, CDPKα(1−328)-VU-1[a]

enzyme	$K_{m_{app}}$ (μM)	$V_{max_{app}}$ (μmol/min/mg)
CDPKα	24.4, 28.6	3.19, 3.46
CDPKα(1−328)-VU-1	37.8, 49.5	3.60, 3.93

[a] Kinetic parameters for enzymes were determined from double-reciprocal analysis of data obtained at an ATP concentration of 60 μM and various concentrations of syntide-2 as peptide substrate. Results are reported from two independent experiments.

Expression vectors that produce either N-terminal His_6-tagged or N-terminal GST-tagged fusion proteins were used. The His_6-tagged proteins were purified by metal chelation and anion exchange chromatography. His_6-CDPKα(1−398) was eluted from Mono Q HR 5/5 with a NaCl gradient, but His_6-CDPKα(1−328), -(1−333), and -(1−339) were recovered in the flow-through fractions. The chromatographic behavior of the latter three proteins corresponded to the deletion of acidic CaM-like domain (calculated pI = 4.2). The purified recombinant enzymes are shown in Figure 2.

Purification of the catalytic domain (residues 1−312) of CDPKα proved to be more difficult. Unlike the other His_6-proteins, His_6-CDPKα(1−312) did not bind to the metal chelation column in the presence of 60 mM imidazole. Also, when this protein was applied to the hydrophobic chromatography resin, phenyl-Sepharose, it could not be eluted unless 6 M urea was employed. However, expression of the catalytic domain as a GST fusion protein allowed its purification by affinity chromatography on glutathione agarose followed by chromatography on Blue-Sepharose, a resin useful for the purification of CDPK (Putnam-Evans et al.,

1990) and other protein kinases (Jeno & Thomas, 1991). Unlike native CDPK, GST-CDPKα(1−312) was not effectively eluted from this resin by 0.2% (w/v) CHAPS, and only 5% of the applied activity was recovered. These observations suggest that the CDPK catalytic domain is more hydrophobic in nature than the full-length enzyme. The specific activity of GST-CDPKα(1−312), which contains the catalytic domain plus 14 residues of the junction domain, was 0.65 μmol/min/mg (41 min^{-1} or 24% of the activity of full-length CDPKα). As reported previously (Harmon et al., 1994; Harper et al., 1994), its activity was not affected by either the presence or absence of calcium. The low specific activity was due in part to the presence of other proteins in the preparation (see lane 8 in Figure 2), some of which might be derived proteolytically from GST-CDPKα(1−312). Other factors contributing to the low specific activity could be the hydrophobic nature of this mutant enzyme, the presence of some of the residues of the junction domain, and/or the inherent instability of the truncated enzyme.

CLD was not expressed as a fusion protein (see Experimental Procedures), because it could be purified successfully by methods (heat treatment and calcium-dependent chromatography on phenyl-Sepharose) used for calmodulin purification. Preparations of purified CLD contained two polypeptides observed in SDS−PAGE (lane 7 in Figure 2). Both polypeptides showed a calcium-dependent mobility shift on gel electrophoresis and could bind phenyl-Sepharose in a calcium-dependent manner (data not shown), therefore both components of CLD are functional. Analysis of recombinant CLD by mass spectrometry revealed that it is proteolytically cleaved during expression between Arg483 and Lys484 (Jorg Heierhorst and Bruce Kemp, personal communication).

Calmodulin-Like Domain Binds to ΔCLD Mutants. If activation of CDPK involves the binding of the CLD to the junction domain, then CLD should bind to CDPK mutants that contain the junction domain. To test this prediction, biotinylated CLD was incubated with nitrocellulose blots of full-length and truncated CDPK in the presence (Figure 3A) or absence (Figure 3B) of calcium. The background staining of the blots incubated with calcium was high relative to that of the blot incubated without calcium, regardless of whether nonfat milk, BSA, or gelatin was used for blocking the membrane. The strongest signals were observed in lanes containing His_6-tagged truncation mutants (ΔCLD) ending at residues 328, 333, 339, and 398, all of which contain the junction domain (lanes 3−6, Figure 3A). Binding of biotin-CLD to these proteins was greatly enhanced in the presence of calcium (compare Figure 3A and B). Weak staining of both GST-CDPKα(1−312) (lane 2) and GST (lane 1) was observed. GST-CDPKα(1−312) was cleaved by digestion with thrombin into GST and CDPKα(1−312) (Figure 2, lanes 8 and 9), and very weak staining of both polypeptide products was observed (Figure 3C). Biotin-CLD did not bind to the calmodulin-like domain (Figure 3, lanes 8), which contains residues 329−508. These results confirm that a CLD-binding site is not located in the calmodulin-like domain. In addition this observation suggests that there is no intermolecular interaction between CLDs of separate molecules of CDPKα. Taken together these results show that a binding site for CLD is present in the junction domain between residues 312 and 328.

Although biotin-CLD bound to ΔCLD mutants of CDPKα, it bound only weakly to full-length enzyme (lanes 7 in Figure 3). This observation agrees with previous experiments in which biotinylated soybean calmodulin failed to bind full-length CDPK (Harmon et al., 1994). In the presence of calcium, the binding site for CLD may not be accessible to biotin-CLD due to steric hindrance and/or occupation of the binding site by the intrinsic calmodulin-like domain. The observation that biotin-CLD is unable to bind full-length CDPK supports the hypothesis that binding of the CLD to the junction domain is intramolecular and not intermolecular. This hypothesis is further supported by the observations that native soybean CDPK (Putnam-Evans et al., 1990) and recombinant CDPKα (data not shown) are monomeric enzymes that do not dimerize in the presence of calcium.

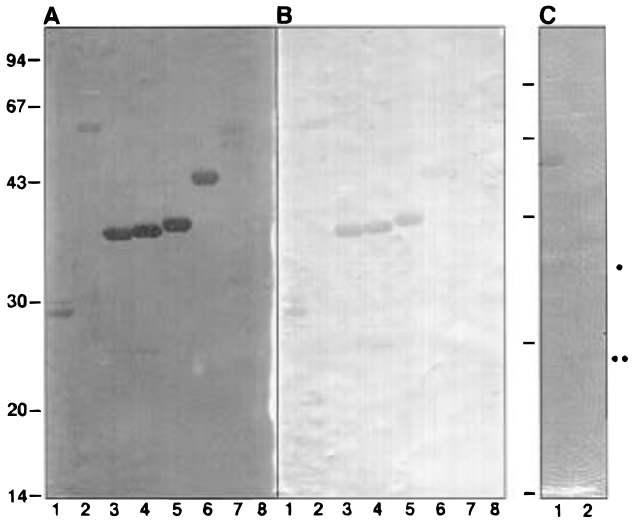

FIGURE 3: Binding of biotinylated CLD by full-length and truncated CDPKα. Proteins (2 μg) were resolved by electrophoresis in a 12% SDS−polyacrylamide gel in the presence of 1 mM EGTA and blotted onto nitrocellulose. The blots were overlaid with biotinylated CLD in the presence of Ca^{2+} (A and C) or EGTA (B) as described in Experimental Procedures. (A, B) Lane 1, GST; 2, GST-CDPKα(1−312); 3, His_6-CDPKα(1−328); 4, His_6-CDPKα-(1−333); 5, His_6-CDPKα(1−339); 6, His_6-CDPKα(1−398); 7, His_6-CDPKα; 8, CLD. (C) Lane 1, GST-CDPKα(1−312); GST-CDPKα(1−312) treated with thrombin for 50 min. The positions of CDPKα(1−312) and GST in lane 2 are indicated by single and double dots, respectively. The molecular mass markers are the same as those described in the legend to Figure 2.

CLD Forms Complexes with Junction Domain Peptides. To further examine the binding of CLD to the junction domain, synthetic peptides corresponding to the junction domain were mixed with CLD and subjected to electrophoresis in non-denaturing glycerol polyacrylamide gels in the presence and absence of 4 M urea. The positively charged synthetic peptides migrated toward the cathode and did not enter the gel (data not shown). Two bands were observed in lanes containing CLD (predicted pI = 4.2 and MW 20 124) (lanes 1, Figure 4). The slower migrating band was an artifact due to oxidation of the CLD, since this band was not present in samples treated with 10 mM DTT (data not shown). Complexes of CLD and basic peptides had mobilities different than either of the two CLD species.

In the presence of Ca^{2+}, the mobility of CLD was not greatly affected by equimolar (100 pmol) CDPKα(302−332), -(310−332), or -(318−332) (Figure 4A, lanes 2, 4, and 6), but a 10-fold molar ratio of the peptides to CLD resulted in the appearance of bands corresponding to complexes and the concomitant diminution of the CLD bands (Figure 4A, lanes 3, 5, and 7). Inclusion of 4 M urea in the gel did not

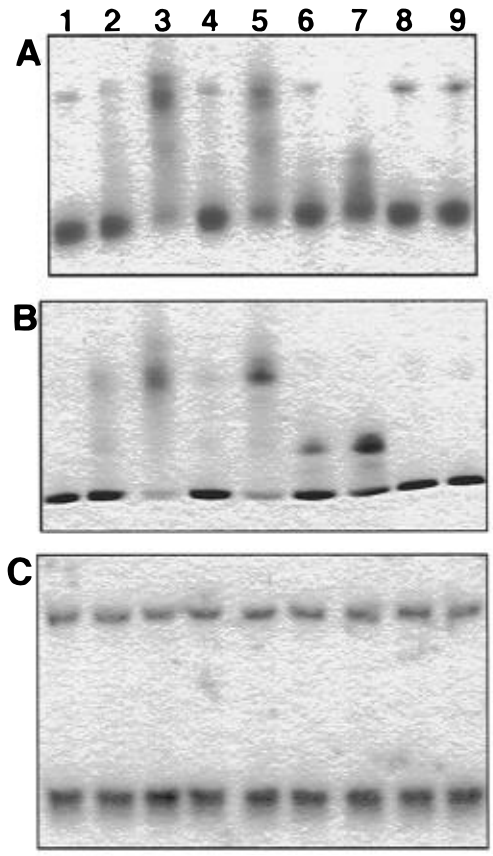

FIGURE 4: Complex formation between CLD and synthetic peptides. CLD (100 pmol, 2 μg) was electrophoresed in non-denaturing gels alone (lane 1) or in the presence of 100 (even lanes) or 1000 pmol (odd lanes) of each of the following peptides: lanes 2 and 3, CDPKα(302−332); lanes 4 and 5, CDPKα(310−332); lanes 6 and 7, CDPKα(318−332); lanes 8 and 9, CDPKα(302−317). (A) $CaCl_2$ (1 mM) was added to all samples and gel buffers, and samples were analyzed by electrophoresis in a glycerol gel. (B) Same as A, except 4 M urea was added to all samples and buffers. (C) Samples were analyzed by electrophoresis in the presence of 2 mM EGTA and 4 M urea.

alter these results (Figure 4B). The greater mobility of the CLD·CDPKα(318−332) complex (Figure 4A and B, lane 7) relative to that of complexes with the other two peptides (lanes 3 and 5) can be attributed to the lower content of basic amino acids in this peptide relative to the others. A fourth peptide, CDPKα(302−317), corresponding to residues in the amino-terminal region of the junction domain, did not form a complex with CLD at either 1:1 or 10:1 molar ratios (Figure 4A and B, lanes 8 and 9). Taken together, these results show that a minimal binding site for CLD is located between residues 318 and 332. These results are in agreement with the biotin-CLD blot overlay experiment (Figure 3) which locates the CLD binding site between residues 312 and 328.

To test whether complex formation was dependent on calcium, mixtures of CLD and peptides were examined in gels containing EGTA. When urea was omitted from the gels, CLD and peptides formed aggregates that did not migrate into the gel (data not shown). Addition of 4 M urea prevented aggregation and allowed entry of the protein into the gel. There was no difference between the pattern of migration in lanes containing CLD alone and lanes containing mixtures of CLD and peptides (Figure 4C). Comparison of these results to those in Figure 4B shows that binding of the peptides to CLD is dependent on the presence of calcium.

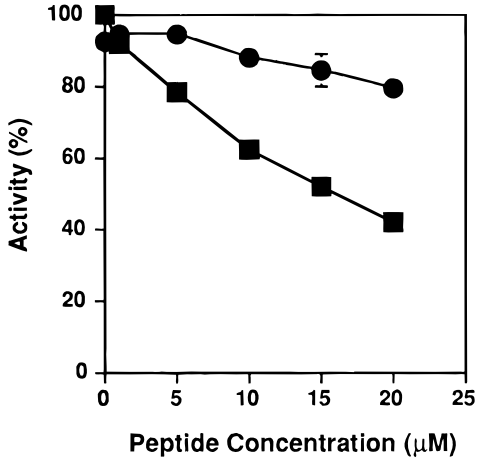

FIGURE 5: Effect of CLD on inhibition of GST-CDPKα(1−312) by peptide 310−332. The activity of GST-CDPKα(1−312) assayed in a mixture containing 15 μM BSA and 100 μM syntide-2 in the presence of calcium was 0.43 μmol min^{-1} mg^{-1}. Activity was measured in the presence (●) or absence (■) of 20 μM CLD and various concentrations of CDPKα(310−332). Data points are the mean and standard deviation of determinations from two independent experiments.

Added CLD Reverses Inhibition of Constitutively Active CDPK by Peptide 310−332. GST-CDPKα(1−312) contains the catalytic domain and a few residues of the junction domain, and it is active in the presence or absence of calcium (Harmon et al., 1994). Consistent with the hypothesis that CDPK is autoinhibited by a pseudosubstrate sequence located in the junction domain, GST-CDPKα(1−312) is inhibited by peptide CDPKα(310−332) which corresponds to the carboxyl end of the junction domain (Figure 5). The inhibition is competitive with respect to syntide-2, and the K_i is 5 μM (Harmon et al., 1994). Since a CLD-binding site is also located between residues 310 and 332 (Figure 5), we asked if inhibition CDPKα(1−312) by peptide CDPKα(310−332) could be reversed by CLD. Addition of 20 μM CLD to an activity assay in which the concentration of inhibitory peptide was varied protected the enzyme from inhibition (Figure 5). The results suggest that binding of CLD and the catalytic domain to peptide 310−332 is mutually exclusive and support the binding model of activation.

CLD Activates ΔCLD Mutants. To test whether CLD can activate CDPKα in trans, the effect of adding CLD to four ΔCLD mutants in the presence or absence of calcium was determined. Since the exact location of the CLD binding site was unknown, three mutants terminating at residues 328, 333, and 339 near the interface between the junction and calmodulin-like domains were tested. The fourth mutant terminated at residue 398 and contained one complete and one partial EF-hand. To control for nonspecific effects of protein concentration in these assays, the total protein was held constant by addition of BSA. In the absence of CLD the activities of His_6-CDPKα(1−328), -(1−333), -(1−339), and -(1−398) were low both in the presence and absence of Ca^{2+} but full-length CDPKα was activated 44-fold by Ca^{2+} (Table 2, −CLD). These results are consistent with previous studies showing that the junction domain (residues 302−332) functions as an autoinhibitory domain (Harmon et al., 1994; Harper et al., 1994). Furthermore, they show that the presence of most of two EF hands is not sufficient for activation of the enzyme by Ca^{2+}. Addition of 20 μM CLD

Table 2: Effect of CLD on the Activity of Full-Length and Mutant CDPKα (min^{-1})[a]

	−Ca^{2+}		+Ca^{2+}	
enzyme	−CLD	+CLD	−CLD	+CLD
His_6-CDPKα	4.2	3.6	157	157
His_6-CDPKα(1−398)	1.9	2.4	0.80	54
His_6-CDPKα(1−339)	0.43	0.81	0.60	5.7
	nd[b]	0.32	nd	2.8
His_6-CDPKα(1−333)	0.76	1.5	0.61	4.4
	0.16	0.80	0.12	8.9
His_6-CDPKα(1−328)	0.39	0.51	0.23	1.2

[a] Specific activities are reported on a molar basis. Activities were determined at a syntide-2 concentration of 200 μM, in the presence of 20 μM CLD (+CLD) or 20 μM BSA (−CLD), and in the presence or absence of Ca^{2+}. Values in each row are for a single enzyme preparation. [b] nd, not determined.

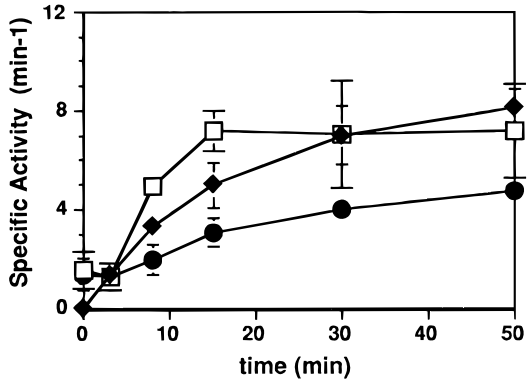

FIGURE 6: Activation of CDPKα and truncation mutants by digestion with trypsin. CDPKα (closed circles), His_6-CDPKα(1−398) (open squares), or His_6-CDPKα(1−328) (closed diamonds) were digested with trypsin as described in Experimental Procedures. Aliquots were removed during the time course of digestion, and the activity in the absence of calcium was determined. Data points are the mean and standard deviation of determinations from two independent experiments.

to full-length CDPKα in the presence or absence of calcium had no effect on activity (Table 2). With the exception of one trial with CDPKα(1−333), addition of CLD in the absence of calcium to the ΔCLD mutants stimulated activity ≤2-fold, while addition of CLD in the presence of Ca^{2+} stimulated activity 5−73-fold (Table 2). While the three shortest mutant CDPKαs achieved only 1%−8% of the specific activity of full-length enzyme, each was stimulated at least 5-fold by the addition of CLD. In experiments with different preparations, His_6-CDPKα(1−398) was consistently activated to a greater extent by addition of CLD than were the mutants in which all four EF hands were deleted (data not shown). In the presence of 20 μM CLD and Ca^{2+}, the specific activity of His_6-CDPKα(1−398) was 40% of that of full-length enzyme.

To examine whether CLD was unable to activate the ΔCLD mutants because they were inherently inactive, we asked if the mutants could be activated by an alternate means. Removal of the junction domain, which is rich in basic residues, by digestion with trypsin should result in an enzyme that is active in the absence of calcium. His_6-CDPKα(1−328), His_6-CDPKα(1−398), and full-length enzyme were digested with trypsin for various times, and their activities were measured in the absence of calcium (Figure 6). All three enzymes had little activity at time zero, but their activities increased during digestion and then leveled off after

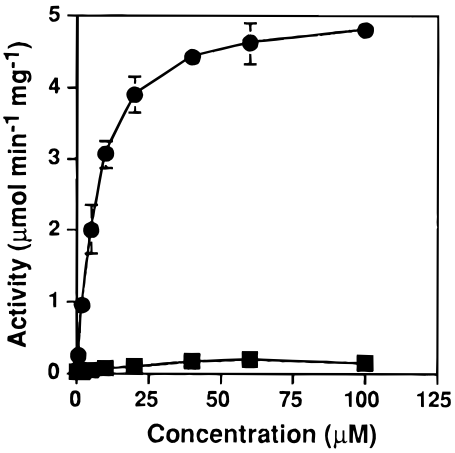

FIGURE 7: Activation of His_6-CDPKα(1−398) by addition of CLD or calmodulin. CLD (circles) or *Arabidopsis* calmodulin (squares) was added to 20 nM His_6-CDPKα(1−398), and the activity of was determined in the presence of calcium as described in Experimental Procedures except that the concentrations of BSA and syntide-2 were 1 mg/mL (15 μM) and 100 μM, respectively. The activity of full-length enzyme in these conditions was 201 min^{-1}. Data are the mean and standard deviations of determinations from two independent experiments.

20 min. Examination of the digested enzymes by SDS−PAGE showed that all three enzymes were converted to single species of 34 kDa after 20 min, and this protein was resistant to further digestion (data now shown). Digested His_6-CDPKα(1−398) and full-length CDPK had ≤6% of the activity of undigested enzymes measured under optimal conditions. His_6-CDPKα(1−328) was activated by digestion to the same degree as His_6-CDPKα(1−398), and this level of activity was higher than that obtained by addition of the CLD to this His_6-CDPKα(1−328) (Table 2). These experiments show that, although His_6-CDPKα(1−328) was capable of activity, CLD was unable to fully stimulate its activity.

Calmodulin is Not as Effective as CLD in Activation of His_6-CDPKα(1-398). The dependence of the activity of His_6-CDPKα(1−398) on the concentration of CLD or *Arabidopsis* calmodulin was determined (Figure 7). At 100 μM CLD the truncated enzyme was activated 480-fold to a specific activity of 4.8 μmol/min/mg, which when compared on a molar basis is 108% of that of full-length enzyme measured in the same conditions. The $K_{0.5}$ for CLD was 7 μM. The high specific activity shown in Figure 7 can be attributed to improved conditions, since when the experiment was performed with the same assay conditions and CLD preparation used for the experiments in Table 2, the maximal activity was 50% (molar basis) of that of full-length enzyme and the $K_{0.5}$ was 12 μM (data not shown). No increase in the activation of His_6-CDPKα(1−328) by CLD was observed when the improved conditions were used. The results in Table 2 and Figure 7 show that the presence of a partial CLD sequence in His_6-CDPKα(1−398) contributes to the ability of added CLD to stimulate activity.

Calmodulin was much less effective than CLD in activation of His_6-CDPKα(1−398). Activity was stimulated 19-fold to a maximal specific activity of 0.19 μmol/min/mg (Figure 7). The $K_{0.5}$ was 20 μM. Similar results were obtained when VU-1 calmodulin was used in place of *Arabidopsis* calmodulin (data not shown). These results suggest that activation of CDPKα and the CDPKα-VU1 chimera occurs by a mechanism other than simple binding.

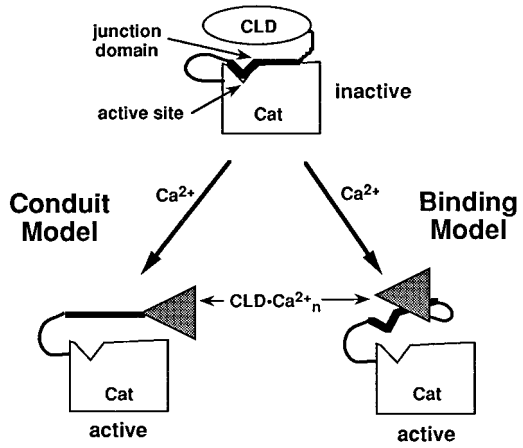

FIGURE 8: Models for regulation of CDPK activity. In both models CDPK is autoinhibited in the absence of Ca^{2+} by the interaction of a pseudosubstrate sequence (V-shaped portion of the junction domain) with the active site. Activation occurs when the binding of Ca^{2+} to the calmodulin-like domain causes a conformational change in the CLD. In the conduit model (left arrow), the conformational change in the CLD affects the conformation of the adjacent junction domain and pseudosubstrate. In the intramolecular binding model (right arrow), the conformational change in the CLD allows it to bind to a binding site located in the junction domain.

DISCUSSION

In this work, we explored whether intramolecular binding of the calmodulin-like domain to the junction domain of soybean CDPKα contributes to activation. The binding model as described in Figure 8 predicts (1) that CLD binds to a site in the junction domain in a calcium-dependent manner and (2) that binding of CLD to the junction domain in the presence of calcium leads to activation of the enzyme. In this model the single event that leads to activation is binding between the CLD and the junction domain, therefore binding and activation should be possible whether or not CLD is attached to the rest of the molecule.

The first prediction was tested by blot overlays of truncation mutants of CDPKα. Biotinylated CLD bound to mutant enzymes containing the junction domain, but it did not bind to CLD on the blot (Figure 3). Our results also show that it bound weakly to the catalytic domain and to GST, and it caused high background staining of blots incubated in the presence of calcium. The data in Figure 5 show that activity of the catalytic domain in the absence of inhibitory peptide was not affected greatly by the addition of CLD. These results suggest that the weak binding of biotin-CLD to the catalytic domain is nonspecific and inconsequential. Binding of CLD to the junction domain was further examined in experiments with synthetic peptide analogs of the junction domain of CDPKα (Figure 4). CLD bound to synthetic peptides containing residues 318−332 but not to the peptide containing residues 302−317. The lack of binding to the latter peptide, which has a p*I* of 9.0, shows that complex formation of CLD with the other peptides is not nonspecific interaction of the basic peptides with the acidic CLD. These data and the results of the blot overlay experiments show that a CLD binding site is located between residues 312 and 328 in the junction domain and fulfill the first prediction of the binding model. The observations of Huang et al. (submitted) with AK1, a CDPK from *Arabidopsis*, are in agreement with our findings. They have shown that the calmodulin-like domain binds to the junction domain with a K_d of 6−7 μM.

Since CLD can bind to a site in the junction domain, the question of whether their interaction is inter- or intramolecular arises. Gel filtration chromatography performed in the presence or absence of calcium shows that active native soybean cell CDPK (Putnam-Evans, 1990) and recombinant CDPKα (data not shown) are monomeric. In addition, isolated CLD does not bind to full-length CDPKα (Figure 3). These observations indicate that intermolecular binding of CLD to the junction domain does not occur and support the idea that the binding is intramolecular.

To test the second prediction of the model, we measured the activity of the truncation mutants in the presence and absence of added CLD in the presence or absence of calcium. Each of the ΔCLD mutants contained all of the junction domain and in the absence of CLD all were inactive regardless of the calcium concentration. Addition of CLD in the presence of calcium stimulated the activity of all the mutants but to various degrees (Table 2). Although CLD could bind to all the ΔCLD mutants (Figure 3), it did not highly activate mutant enzymes missing all of the calmodulin-like domain. Since the truncated enzymes could be activated by treatment with trypsin, the low degree of activation by CLD was not due to the inherent inactivity of the mutants. These data suggest that binding of CLD to the junction domain is not sufficient for complete activation.

Added CLD highly activated the truncation mutant His_6-CDPKα(1−398) that retained nearly half of the CLD. Neither calcium alone nor CLD alone activated this mutant, but CLD plus calcium activated it up to 450-fold (Figure 7). It is possible that the conformation of the junction domain is different in His_6-CDPKα(1−398) than in the enzymes terminating at residues 298, 333, or 339. This difference in conformation could arise from the presence of additional polypeptide at the carboxyl-terminal end of the CLD-binding site, or it could be caused by a conformational change in the EF-hand(s) present in His_6-CDPKα(1−398). A conformational change in the junction domain could either promote the binding of CLD or weaken the binding of the pseudosubstate to the active site. An alternative possibility is that the CLD binding site, which would be located at the carboxyl-terminus of the shorter ΔCLD mutants, could be subject to proteolysis during expression. The latter possibility is unlikely because the mutant enzymes migrated at positions in SDS−PAGE consistent with their predicted sizes, and all of them bound biotin-CLD (Figure 3).

Half-maximal activation of His_6-CDPKα(1−398) occurred at a CLD concentration of 7 μM (Figure 7), and this $K_{0.5}$ is in agreement with the K_d of 6−7 μM for the binding of CLD and the junction domain determined by Huang et al. (submitted). These data show that CLD has a lower affinity for its binding site in CDPK than does calmodulin for its binding sites in target enzymes. The binding constant of CaM with NAD kinase or MLCK is ≤1 nM and is 25−100 nM with CaMPKII (Hanson & Schulman, 1992; Roberts & Harmon, 1992). The apparent low affinity of CLD for binding the junction domain does not preclude binding from contributing to activation, however, since the CLD is covalently attached to CDPK and the low binding affinity may be overcome by the high local concentration of each component.

The sequences of calmodulin and CLD are only 40% identical, and CLD is unable to activate the calmodulin-dependent enzymes, cyclic nucleotide phosphodiesterase (PDE) (Harmon et al., 1995), and CaMKII (data not shown). CLD does not prevent the activation of PDE by calmodulin, suggesting that CLD does not bind to this enzyme (Harmon et al., 1995). In contrast to these observed functional differences, calmodulin can substitute for CLD in the chimeric enzyme CDPKα-VU-1 and can support full, calcium-dependent activity (Table 1). Since both CLD (Figures 3 and 4) and CaM (Harmon et al., 1995) bind to the junction domain peptides, it is possible that this is the property that allows both CLD and CaM to activate CDPKα and the chimera. However, CLD, but not calmodulin, significantly activated His_6-CDPKα(1−398) (Figure 7). If the binding model (Figure 8) were correct, then both CLD and CaM should activate His_6-CDPKα(1−398). The paradox that calmodulin is able to bind to the junction domain and to function in the activation of the chimera but is unable to activate His_6-CDPKα(1−398) suggests that the simple binding model is not valid.

Our data suggest that intramolecular binding between the calmodulin-like domain and a binding site in the junction domain occurs, but that binding is not sufficient for activation of CDPK. Interaction between CLD and additional parts of the enzyme may contribute to activation. Further experiments with the chimera and mutant enzymes employing site-directed mutagenesis should give insight into the mechanism, but definitive proof will most likely require the determination of crystallographic structures of CDPK.

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