Pseudosubstrate Inhibition of CDPK, a Protein Kinase with a Calmodulin-like Domain†

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ABSTRACT: Between the catalytic and regulatory domains of calmodulin-like domain protein kinase, CDPK, is a junction domain which has some identity to the autoinhibitory domain of calmodulin-dependent protein kinase type II (Harper, J. F., Sussman, M. R., Schaller, G. E., Putnam-Evans, C., Charbonneau, H., & Harmon, A. C. (1991) *Science 252*, 951–954). To investigate whether CDPK's junction domain also functions as an autoinhibitory domain, we determined the effect of synthetic peptides, corresponding to sequences within the junction domain, on the activity of native soybean CDPK. Three peptides, corresponding to residues 310-332, 318-332, 302-317, were competitive inhibitors with respect to syntide-2 and had K_i values of 5, 25, and 85 μM, respectively. These peptides were uncompetitive inhibitors with respect to ATP and had K_i values of 24, 220, and 510 μM, respectively. A fourth peptide, CDPKα 302-332, inhibited activity by a mixed mechanism with respect to both syntide-2 (K_i = 1.9 μM; K'_i = 5.0 μM) and ATP (K_i = 15 μM; K'_i = 4.5 μM). Three of the peptides, CDPKα 302-332, 310-332, and 318-332, formed complexes with soybean calmodulin during electrophoresis in native polyacrylamide gels and were able to inhibit calmodulin-dependent protein kinases. Given the similarity between CDPK's calmodulin-like domain and calmodulin (40% sequence identity), it was possible that these peptides could inhibit activity through interaction with the calmodulin-like domain rather than the catalytic domain. To address this possibility, a cDNA encoding the first 312 residues of soybean CDPKα was constructed and expressed in *Escherichia coli*. This enzyme, which is missing most of the junction domain and all of the calmodulin-like domain, was active in the presence and absence of calcium. Peptide CDPKα 310-332 inhibited this truncated enzyme competitively with respect to syntide-2 (K_i = 4 μM). These results show that the junction domain is capable of functioning as an autoinhibitory domain, possibly through a pseudosubstrate site located between residues 310 and 332.

Calcium-dependent protein kinase (CDPK[1]) belongs to a new class of calcium-regulated protein kinases having regulatory domains similar in sequence to calmodulin [reviewed in Roberts (1993) and Roberts and Harmon (1992)]. The more descriptive name "calmodulin-like domain protein kinase", also abbreviated CDPK, has been adopted (Roberts, 1993). CDPK activity is found in numerous plant species and organs and in several subcellular compartments (Roberts & Harmon, 1992). Deduced amino acid sequences for CDPKs from soybean, carrot, *Arabidopsis*, rice, corn, and the protozoan *Plasmodium* have been reported (Harper et al., 1991, 1993; Kawasaki et al., 1993; Poovaiah & Reddy, 1993; Suen & Choi, 1991; Zhao et al., 1993). CDPK purified from soybean suspension cultures was the first of these enzymes to be described (Harmon et al., 1987; Putnam-Evans et al., 1990). Its activity is stimulated 50–100-fold by the direct binding of Ca^{2+} (Harmon et al., 1987). CDPKα is encoded by the soybean cDNA SK5 and has a predicted molecular mass of 57 kDa (Harper et al., 1991). A CDPK from *Arabidopsis*, AK1, has an amino-terminal domain that is 113 residues longer than that of CDPKα and a predicted mass of 72 kDa (Harper et al., 1993). These two enzymes are 70% identical in their overlapping regions, and both contain a regulatory domain near the carboxyl terminus that is 40% identical to that of spinach calmodulin and a catalytic domain near the amino terminus that is 39% identical to that of calcium/calmodulin-dependent protein kinase type II (CaMKII).

CDPK purified from soybean cells is similar to calmodulin in several characteristics (Harmon et al., 1987; Putnam-Evans et al., 1990). CDPK binds calcium directly and has a $K_{0.5}$ of 2 μM. The mobility of both calmodulin and CDPK in SDS–polyacrylamide gels is greater in the presence of calcium than in the presence of EDTA. The naphthalene sulfonamide drug W7 and trifluoperazine inhibit the activity of both CDPK and calmodulin. In addition, both proteins bind to phenyl-Sepharose in a calcium-dependent manner. These observations suggest that the calmodulin-like domain of CDPK and calmodulin have similar biochemical properties.

The similarity in sequence between soybean CDPKα and rat brain CaMKII extends into the region between the catalytic domain and the calcium-binding domain (Harper et al., 1991). In this region, there is a stretch of 15 residues, of which eight are identical (Figure 1). This region of CaMKII is important for autoregulation [reviewed in Colbran et al. (1989a)]. Another calmodulin-dependent enzyme, myosin light chain kinase (MLCK) from skeletal or smooth muscle, also contains an autoinhibitory domain which turns the enzyme off when calcium and calmodulin are absent. Autoinhibition is ac-

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[1] Abbreviations: CDPK, calmodulin-like domain protein kinase; CDPKα, protein encoded by soybean cDNA SK5; CaMKII, calcium/calmodulin-dependent protein kinase type II; PMSF, phenylmethylsulfonyl fluoride; MLCK, myosin light chain kinase; MLCKI, myosin light chain kinase inhibitor peptide; CaM, calmodulin.

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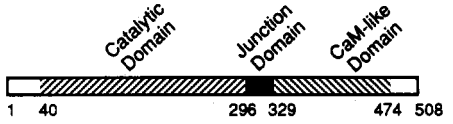

B

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                    .. . . ... .
CDPKα302-332    PLDSAVLSRLKQFSAMNKLKKMALRVIAERL
CAMKIIα281-309  MHRQETVDCLKKFNARRKLKGAILTTMLA
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FIGURE 1: Functional domains of CDPKα. (A) The relationship between the functional domains of CDPKα is shown. The protein kinase catalytic domain is located near the amino terminus, the calmodulin-like domain is located near the carboxyl terminus, and the junction domain, which has similarity to the autoinhibitory domain of CaMKII, is located between the other two domains. (B) Residues 302-332 of CDPKα, which are in the junction domain and beginning of the CaM-like domain, are aligned with the autoinhibitory domain of the α subunit of CaMPKII. Identical residues in CDPKα and CaMKII are indicated by ●. The minimal Ca^{2+}/CaM-binding domain (residues 296-309) of CaMKII (Hanley et al., 1988; Payne et al., 1988) is underlined, and the regulatory Ca^{2+}/CaM-dependent autophosphorylation site (Thr286), responsible for the generation of Ca^{2+}/CaM-independent form of CaMKII (Miller et al., 1988; Schworer et al., 1988; Thiel et al., 1988), is double-underlined. Sequences important for ATP-directed inhibition (281-289) and for protein/peptide substrate-directed inhibition (290-302) (Smith et al., 1992) are indicated by * and ^, respectively.

complished through the binding of a pseudosubstrate site within the autoinhibitory domain to the active site and prevention of binding of the peptide substrate. In addition to interfering with peptide binding, the autoinhibitory domain of CaMKII interferes with the binding of ATP (Smith et al., 1992). Activation occurs when Ca^{2+}/calmodulin binds to the autoinhibitory domain in a region that overlaps or is very close to the pseudosubstrate site.

Since it has been suggested that the gene encoding calcium-dependent protein kinase resulted from the fusion of two genes, one encoding a calcium/calmodulin-dependent protein kinase and the other a calmodulin-like protein (Harper et al., 1991), it will be interesting to see if the mechanism of autoregulation of CDPK is similar to that of the calmodulin-dependent protein kinases. This study was undertaken to investigate whether residues 302-332 in the junction domain of CDPKα function as an autoinhibitory domain. To determine whether this region contains a pseudosubstrate site, synthetic peptides were tested as inhibitors of CDPK purified from soybean cells. In addition, CDPKα truncated at residue 312 was expressed in *Escherichia coli* and characterized.

EXPERIMENTAL PROCEDURES

Materials. The following synthetic peptides were purchased from BACHEM California (sequences of peptides are given in parentheses): residues 281-309, which are the autoinhibitory domain, from the α subunit of calmodulin-dependent protein kinase II (MHRQETVDCLKKFNARRKLKGAILTTMLA) (Colbran et al., 1988); autocamtide-2 (KKALRRQETVDAL), which contains the regulatory autophosphorylation site of CaMKII (Hanson et al., 1989); H1-7 (RRKASGP), which is a histone H1 phosphorylation site (Pomeranz et al., 1977); myosin light chain kinase inhibitor (KRRWKKNFIAV) (Pearson et al., 1991); skeletal muscle myosin light chain kinase substrate (AKRPQRATSNVFS) (Michnoff et al., 1986); smooth muscle myosin light chain kinase substrate (KKRAARATSNVFA) (Kemp & Pearson, 1985); and syntide-2 (PLARTLSVAGLPGKK) (Hashimoto & Soderling, 1987).

Peptides synthesized by the Protein Chemistry Core Laboratory[2] at the University of Florida included syntide-2 and four peptides corresponding to sequences within CDPKα: 302-332 (PLDSAVLSRLKQFSAMNKLKKMALRVIAERL), 310-332 (RLKQFSAMNKLKKMALRVIAERL), 318-332, (NKLKKMALRVIAERL), and 302-317 (PLDSAVLSRLKQFSAM). Norleucine was substituted for methionine (M) in these peptides. Synthetic peptides were used either without further purification or after purification by HPLC with no difference in results.

Skeletal muscle myosin light chain kinase was a gift of Peter Kenelly, Virginia Polytechnic Institute and State University. Recombinant α subunit of mouse brain calcium/calmodulin-dependent protein kinase II was a gift of Thomas Soderling, Vollum Institute, Oregon Health Sciences Center, Portland, OR.

Soybean cell cultures (*Glycine max* L.) were obtained from Dr. C. J. Lamb, Salk Institute, and were maintained as described previously (Norman et al., 1986). Soybean cells were harvested during log-phase growth by collection on Miracloth (Calbiochem), drained of culture media, and washed with 20 mM Tris, 0.4 M sorbitol, and 10 mM $MgCl_2$ (pH 8.5). Washed cells were frozen by dropping aliquots into liquid nitrogen and stored at −80 °C.

Protein Purification. Calcium-dependent protein kinase was purified from frozen soybean cell cultures by a modification of the method of Putnam-Evans et al. 1990). All steps were performed at 4 °C. One kilogram of frozen cells was homogenized in 20 mM Tris (pH 7.2), 2.5 mM EDTA, 1 mM PMSF, 10 μg/mL leupeptin, and 20 μg/mL aprotinin in five bead beater apparatuses (BioSpec Products, Inc.) at 4 °C. For each run of an apparatus, 90 g of cells, 140 mL of buffer, and 200 g of 0.5-mm, acid-washed glass beads were combined, and the power was turned on for 1 min, off for 3 min, and then on again for 1 min. Homogenates were decanted from the beads, and after all the cells had been processed, the beads were washed with buffer. A volume of 3 L of buffer was used, and the volume of the homogenate was 4 L. After cellular debris was pelleted by centrifugation at 11 000*g* for 45 min, the supernatant was passed over a column of DEAE–cellulose as previously described (Putnam-Evans et al., 1990). Eluate fractions containing CDPK activity were pooled, and solid ammonium sulfate was added to 60% saturation. The mixture was stirred for 1 h, and precipitated protein was pelleted by centrifugation at 15 000*g* for 20 min. The pellets were resuspended in 200 mL of 20 mM Tris (pH 7.2) and stirred for 30 min. The concentration of calcium was brought to 0.5 mM by addition of 1 M $CaCl_2$. The pH was monitored during the addition and was kept at 7.2 by the addition of 1 M Tris (pH 8.0). The solution was stirred for 1 h and then clarified by centriguation at 15 000*g* for 30 min. The supernatant was chromatographed on phenyl-Sepharose as previously described. Fractions containing CDPK activity were pooled and concentrated to 1 mL in an Amicon stirred ultrafiltration cell with a YM-10 membrane. The concentrate was chromatographed on a Superose 12 HR10/30 gel filtration column on a Pharmacia FPLC system. Fractions containing CDPK

[2] Funding for this facility was received from an NIH Shared Instrumentation Grant, and it is supported by funds from the Interdisciplinary Center for Biotechnology Research, Department of Biochemistry and Molecular Biology, Division of Sponsored Research, and the College of Medicine.

activity were pooled and chromatographed on Blue-Sepharose as previously described (Putnam-Evans et al., 1990).

Calmodulin was purified from 1 kg of frozen soybean cells by a modification of the method of Watterson et al. (1980). Soybean cells were homogenized in 50 mM Tris (pH 8.0), 1 mM EGTA, 1 mM 2-mercaptoethanol, 1 mM PMSF, 10 μg/mL leupeptin, and 20 μg/mL aprotinin in bead beaters as described above. The homogenate was clarified by centrifugation at 11 000*g* for 45 min. Solid ammonium sulfate was added to the supernatant to 55% saturation, and the solution was stirred for 1 h. Following centrifugation of the mixture at 10 000*g* for 1 h, calmodulin was precipitated from the supernatant by adjustment of the pH to 4.1 by addition of 50% v/v sulfuric acid. The mixture was stirred for 1 h, and precipitated protein was collected by centrifugation at 10 000*g* for 1.5 h. The pellet was dissolved in 10 mM Tris, pH 7.5, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 200 mM NaCl. The pH was adjusted to 7.4, and the mixture was dialyzed overnight against the same buffer. The solution was clarified by centrifugation at 40 000*g* for 1 h and loaded on a column of Q-Sepharose fast flow. Calmodulin was eluted by a linear gradient of 0.2–0.5 M NaCl. Fractions containing calmodulin were identified by native gel electrophoresis, pooled, dialyzed versus deionized water, and lyophilized. The dry powder was dissolved in 10 mM Tris (pH 7.2), 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol, and 2 mM $CaCl_2$ and loaded on a column of phenyl-Sepharose equilibrated in the same buffer. After being washed with buffer containing 0.2 M NaCl, calmodulin was eluted with 10 mM Tris (pH 7.2) and 5 mM EDTA. To achieve final purification, calmodulin was chromatographed on Superose 12 equilibrated in 100 mM phosphate buffer (pH 7.4). Soybean calmodulin was able to fully activate myosin light chain kinase.

Calmodulin Biotinylation and Overlay. Calmodulin was biotinylated as described by Billingsley et al. 1985) with Biotin-X-NHS (Calbiochem). Biotinylated calmodulin was able to fully activate myosin light chain kinase. Proteins were separated by electrophoresis in SDS–polyacrylamide gels in the presence of 5 mM EGTA, blotted to nitrocellulose, and then tested for calmodulin binding by the method of Colbran (1993), except that 1 μM biotinylated soybean calmodulin was used and either 1 mM $CaCl_2$ or 5 mM EGTA was included in the incubation buffers. Biotinylated calmodulin was detected with ExtrAvidin–alkaline phosphatase conjugate (Sigma) at a 1:1000 dilution.

Enzyme Assays. Calcium-dependent protein kinase assays were performed in a total volume of 50 μL in 0.6-mL microfuge tubes containing 50 mM HEPES (pH 7.2), 10 mM $MgCl_2$, 5 mM EDTA, 3 mM $CaCl_2$, 0.1 mg/mL BSA, 3 nM CDPK, the indicated amount of peptide substrate, and the indicated concentration of [γ-^{32}P]ATP (500 cpm/pmol). Assay tubes were incubated for 6 min at 30 °C prior to initiation of the reaction by addition of ATP. Reactions were terminated after 6 min by spotting 10 μL of the reaction mixture onto circles of P81 paper. The papers were washed five times in 150 mM H_3PO_4, once in 95% ethanol, air-dried, and counted in Scintiverse BD in a liquid scintillation counter.

Myosin light chain kinase (MLCK) activity was assayed in a volume of 25 μL containing 50 mM HEPES (pH 7.0), 0.5 mM DTT, 12.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.5 mg/mL BSA, 0.1% (v/v) Triton X-100, 2 μM calmodulin (saturating concentration) or 20 nM calmodulin (subsaturating concentration), 0.2 mM skeletal muscle myosin light chain kinase substrate peptide, 0.25 mM [γ-^{32}P]ATP (500 cpm/pmol), and 2 ng of rabbit skeletal muscle MLCK for 6 min at 30 °C.

The activity of recombinant calcium/calmodulin protein kinase II was measured in a volume of 25 μL containing 50 mM HEPES (pH 7.5), 10 mM magnesium acetate, 0.5 mM calcium chloride, 2 μM calmodulin (saturating concentration) or 20 nM calmodulin (subsaturating concentration), 20 μM syntide-2, 0.4 mM [γ-^{32}P]ATP (500 cpm/pmol), and 0.1 mU of enzyme for 1 min at 30 °C.

Protein Assay. Concentrations of peptides were calculated from amino acid compositions determined by the Protein Sequencing Core Facility, University of Florida. Concentrations of calmodulin were determined from absorbance measurements using $E^{1\%}_{276\,nm}$ of 0.09. Concentrations of calcium-dependent protein kinase were determined by the Bio-Rad dye-binding assay, based on the method of Bradford (1976).

Electrophoresis. Denaturing polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970). Native gel electrophoresis was performed according to the method of Watterson et al. (1976) with either 1 mM $CaCl_2$ or 1 mM EGTA added to all the buffers.

Expression of Recombinant CDPK in E. coli. A cDNA clone encoding the full length of soybean CDPK was constructed from two plasmids containing overlapping cDNAs: pBluescript SK5 encoding the 5′ region and pBluescript 3′ encoding the 3′ region (Harper et al., 1991). *Eco*RI-digested SK5 was ligated into the single *Eco*RI site of pBluescript 3′. The resulting plasmid, pBluescript AY, was used as the target sequence for the introduction of an engineered *Nde*I site at the initiating methionine codon by polymerase chain reaction (PCR) amplification. The primer GGTCCCATATGGCTGCGAAATCTAGTTCG was used for the 5′ end of the coding sequence (underlined residues show the *Nde*I site at the initiating methionine codon). The primer of the second strand at the 3′ end was 5′GGTCCATCGATTTATTACTTAAAGTAGCCC (underlined residues show the *Cla*I site). The PCR product was digested with *Nde*I and *Cla*I, and the resulting fragment was inserted into the corresponding site of the pETh-3a vector, which was derived from pET-3a (Studier et al., 1990) by the addition of multicloning sites and was a gift of T. Hattori and D. R. McCarty, University of Florida. The resulting plasmid was named pET1530. A second plasmid, pHis1530, which expresses full-length CDPK with six histidine residues added at the amino terminus, was constructed by insertion of the *Nde*I–*Bgl*II fragment from pET1530 into the *Nde*I–*Bam*HI site of pET-15b (Novagen). The integrity of the DNA inserts was determined by DNA sequencing. The parent cDNA for all constructs contained four base changes, but each occurred at the "wobble" position in its respective codon and did not cause a change in the amino acid sequence of the predicted protein. No other differences between the sequences of our constructs and the published sequence for CDPKα (Harper et al., 1991) were found.

A plasmid, pHis312, encoding residues 1-312 of CDPKα was constructed by PCR amplification with pAY as the template. The 5′ and 3′ primers were GGCCTCTAGACCATATGGCTGCGAAATCTAG and CGCGGATCCTTATTTCAGACGTGATAAAAC, respectively. The PCR products were digested with *Nde*I and *Bam*HI and inserted into pET-15b digested with the same enzymes. A second plasmid encoding CDPKα 1-312, p312MQI, was constructed by insertion of the *Nde*I and *Xmn*I fragment of pET1530 into the *Nde*I/*Eco*RV site of pETh-3b. The expressed protein contains residues 1-312 plus three extra amino acids (MQI) at the carboxyl terminus. Both of these constructs were verified by DNA sequencing.

Expression of the plasmids in *E. coli* BL21 (DE3) was induced with IPTG, and active full length or truncated enzyme was recovered from the soluble fraction of extracts or partially purified by ion-exchange chromatography.

Expression and Purification of His_6-CDPKα 1-312. Cells induced to express His_6-CDPKα 1-312 were pelleted and resuspended in nickel column binding buffer (20 mM Tris, 0.5 M NaCl, 5 mM imidazole, pH 8.0). Cells were broken by sonication, and the extract was clarified by centrifugation. The supernatant was loaded onto the nickel column (Sigma) equilibrated with binding buffer. The column was washed with 10 column volumes of binding buffer and then with 8 column volumes of wash buffer (20 mM Tris, 0.5 M NaCl, 60 mM imidazole, pH 8.0). The bound protein was eluted with elution buffer (20 mM Tris, 0.5 M NaCl, 300 mM imidazole, pH 8.0). The activity of the kinase was found in fractions from the second wash. The fraction containing kinase activity was dialyzed against 20 mM Tris, pH 8.0, 2 mM EDTA, and 0.3% 2-mercaptoethanol. The dialyzed sample was applied to a Mono-Q, HR 5/5 column (Pharmarcia) equilibrated with buffer A (20 mM Tris (pH 8.0) and 2 mM EDTA) and eluted with a gradient of NaCl in buffer A. DTT was added to a final concentration of 1 mM to the fraction containing peak activity. This fraction was stored at −70 °C and used in the assay.

Data Analysis and Graphics. Kinetic data were analyzed by a nonlinear least-squares curve-fitting program written in BASIC by John E. Wampler, Department of Biochemistry, University of Georgia. The program follows the algorithm of Marquardt (1963) as described by Bevington (1969). Equations for competitive, uncompetitive, noncompetitive, and mixed inhibition were used for curve fitting, and the computed lines from the equation giving the lowest reduced χ^2 statistic were plotted in the figures. In cases where two equations gave the same value of χ^2, the model employing the smaller number of kinetic parameters was chosen (e.g., competitive inhibition was favored over mixed inhibition). Values for K_i and K'_i were determined from secondary plots of slope and intercept, respectively, versus inhibitor concentration.

Secondary structure predictions were performed with the DNASTAR PROTEAN program. Default settings were used for the Garnier–Robson (Garnier et al., 1978), Chou–Fasman (Chou & Fasman, 1974), and Eisenberg (Eisenberg et al., 1984) methods.

Digital images of polyacrylamide gels and nitrocellulose blots were obtained by scanning them with an HP ScanJet connected to a Macintosh computer. The images were cosmetically retouched and the contrast enhanced without alteration of data with Adobe Photoshop.

RESULTS

Choice of Syntide-2 as Substrate for Kinetic Analysis. Histone IIIS, a lysine-rich histone preparation, is a good substrate for CDPK and was used for the development of the purification procedure (Putnam-Evans et al., 1990). However, histone IIIS is a mixture of polypeptides, several of which are phosphorylated by CDPK, and possible variation in peptide composition from batch to batch makes it unsuitable as a substrate for repeated kinetic analyses. For this reason, synthetic peptides containing the sequence basic-X-X-Ser/Thr, previously identified to be a sequence motif preferentially phosphorylated by CDPK (Roberts & Harmon, 1992), were tested. Syntide-2 (PLARTLSVAGLPGKK; phosphorylation motif is underlined) and autocamtide-2 (KKALRRQETVDAL) both supported high CDPK activity (Table 1). The peptides AKRPQRATSNVFS and KKRAARATSNFA, substrate peptides of skeletal and smooth muscle myosin light chain kinases, respectively, were phosphorylated at only 10–20% of the extent of syntide-2 phosphorylation when each was assayed at a concentration of 100 μM. The peptide H1-7 (RRKASGP) containing a basic-basic-basic-X-Ser sequence, was not phosphorylated by CDPK. The apparent K_m values for autocamtide-2 and syntide-2 were similar, but the apparent V_{max} was higher with syntide-2 than with autocamtide-2 (Table 1). Syntide-2 was chosen for use in kinetic analysis.

Table 1: Kinetic Parameters for Substrates of Soybean Calcium-Dependent Protein Kinase

substrate	$K_{m\ app}$ (μM)	$V_{max\ app}$ (μmol/min/mg)	n
syntide-2[a]	24 ± 6	9.3 ± 1.1	8
autocamtide[a]	29 ± 14	2.8 ± 0.6	3
ATP[b]	19 ± 6	9.3 ± 2.4	5

[a] Kinetic parameters for peptide substrates were determined at an ATP concentration of 60 μM. [b] Parameters for ATP were determined at a syntide-2 concentration of 200 μM.

Inhibition of CDPK Activity with Respect to Syntide-2. If residues 302-332, located between the catalytic and regulatory domains of CDPKα (Figure 1), contain a pseudosubstrate site, then synthetic peptides corresponding to this domain should inhibit activity. Four synthetic peptides corresponding to the putative autoinhibitory domain (Table 2) were tested for ability to inhibit CDPK with respect to the peptide/protein substrate. Kinetic assays were performed with native soybean CDPK, a fixed ATP concentration of 60 μM, and various concentrations of syntide-2 (25–100 μM). Control experiments showed that the CDPK peptides did not interfere with the binding of phosphorylated syntide-2 to the phosphocellulose paper.

The results of assays performed in the presence of 2.5–10 μM CDPKα 302-332 are shown in the double reciprocal plot in Figure 2A. Analysis of the data by a nonlinear curve-fitting program showed that it fit a mixed inhibition model best. The lines in the graph are a double reciprocal representation of the best-fit model. K_i and K'_i were 1.9 ± 0.5 and 5.0 ± 0.6 μM, respectively, for four determinations. When histone IIIS was used in place of syntide-2, qualitatively similar results were obtained (data not shown).

To determine whether specific regions within the 302-332 sequence were important for inhibition, three shorter peptides were tested. CDPKα 302-317 was analyzed at concentrations ranging from 50 to 200 μM (Figure 2B). Analysis of the data by a nonlinear curve-fitting program indicated that the inhibition is competitive with syntide-2 (Figure 2B). K_i was 85 ± 17 μM for three determinations. CDPKα 310-332, tested in the range of 5–20 μM, was competitive with syntide-2 (Figure 2C). K_i was 4.8 ± 1.7 μM for four determinations. CDPKα 318-332, tested in the range of 25–100 μM, was also competitive with syntide-2 (Figure 2D) and had a K_i of 25 ± 10 μM for four determinations. Since the two nonoverlapping peptides 302-317 and 318-332 were both competitive inhibitors, at least two sequences within the region are capable of interfering, directly or indirectly, with the peptide binding site, and one of the peptides is an order of magnitude more potent than the other. However, as shown above, the type of inhibition changes from competitive to mixed when the peptide is extended in length, thus CDPKα 302-332 inhibits by interacting with a site other than the peptide binding site.

CDPK Does Not Phosphorylate the Inhibitory Peptides. Three of the inhibitory peptides contain the potential phos-

Table 2: Effect of Synthetic Peptides on the Activity of CDPK Purified from Soybean Cells

CDPKα peptide	sequence	syntide-2 inhibition	syntide-2 K_i (μM)	syntide-2 K'_i (μM)	ATP inhibition	ATP K_i (μM)	ATP K'_i (μM)
302-332	PLDSAVLSRLKQFSAMNKLKKMALRVIAERL	mixed	1.9 ± 0.5	5.0 ± 0.6	mixed	15 ± 2.8	4.5 ± 0.6
310-332	RLKQFSAMNKLKKMALRVIAERL	competitive	4.8 ± 1.7	NA[a]	uncompetitive	24 ± 8	NA
318-332	NKLKKMALRVIAERL	competitive	25 ± 10	NA	uncompetitive	220 ± 13	NA
302-317	PLDSAVLSRLKQFSAM	competitive	85 ± 17	NA	uncompetitive	510 ± 13	NA

[a] Not applicable.

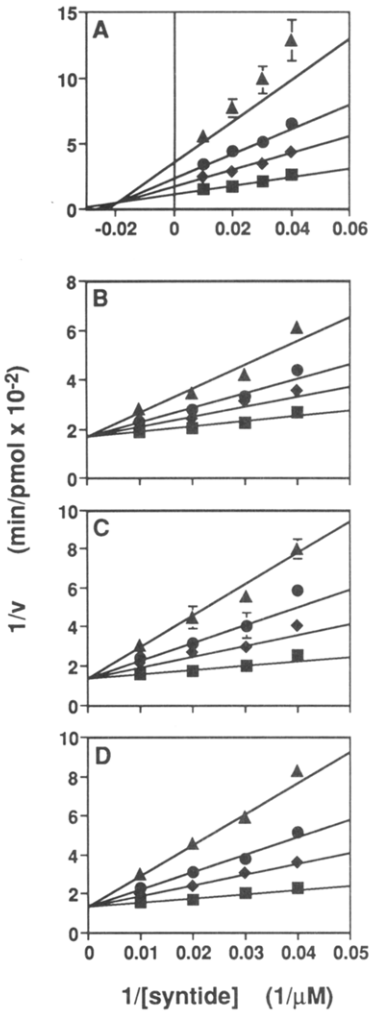

FIGURE 2: Effect of synthetic peptides on CDPK activity measured at constant ATP concentration and various syntide-2 concentrations. Activity was measured with 60 μM ATP and 25, 33, 50, or 100 μM syntide-2. (A) CDPKα 302-332 at 0 (■), 2.5 (◆), 5 (●), and 10 (▲) μM. (B) CDPKα 302-317 at 0 (■), 50 (◆), 100 (●), and 200 (▲) μM. (C) CDPKα 310-332 at 0 (■), 5 (◆), 10 (●), and 20 (▲) μM. (D) CDPKα 318-332 at 0 (■), 25 (◆), 50 (●), and 100 (▲) μM. Error bars indicate standard deviations.

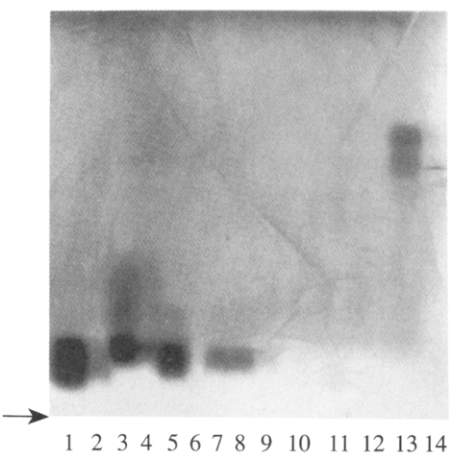

FIGURE 3: Analysis of substrate peptides by SDS–polyacrylamide gel electrophoresis. Peptides (100 μM) and histone IIIS (0.5 mg/mL) were phosphorylated in the presence (odd lanes) or absence (even lanes) of Ca^{2+} in the standard assay mixture. Reactions were terminated by addition of an equal volume of Laemmli SDS sample buffer and boiled. Samples (10 μL) were resolved in a 15% SDS–polyacrylamide gel. The gel was immediately autoradiographed upon completion of electrophoresis and was not stained. The arrow indicates the position of the dye front. The samples were syntide-2 (lanes 1, 2), autocamtide-2 (lanes 3, 4), skeletal muscle myosin light chain kinase peptide (lanes 5, 6), smooth muscle myosin light chain kinase peptide (lanes 7, 8), H1-7 (lanes 9, 10), CDPKα 302-317 (lanes 11, 12), and histone IIIS (lanes 13, 14).

phorylation site KQFS (residues 312-315) having the basic-X-X-S motif. Since two of the peptides, 302-317 and 310-332, inhibited activity competitively with respect to the peptide substrate, it was important to determine whether the peptides were phosphorylated by CDPK. All four inhibitor peptides were tested as substrates at a concentration of 100 μM in the standard assay, but none was phosphorylated significantly (<1% compared to syntide-2). The CDPK peptides are basic and are expected to bind to P81 paper. But since a negative result could result from lack of binding, the peptides were also examined by electrophoresis in SDS–polyacrylamide gels (Figure 3). To prevent leaching of the peptides from the gels, the wet, unstained gels were immediately autoradiographed upon completion of electrophoresis. Phosphorylation of syntide-2, autocamtide-2, skeletal and smooth muscle MLCK substrate peptides, and histone IIIS was stimulated by Ca^{2+}, but H1-7 and CDPKα 302-317 were not phosphorylated. Similar analysis of CDPKα 302-332 and 310-332 showed that these peptides were not phosphorylated (data not shown). The relative intensities of the signals for the substrate peptides and proteins (Figure 3) agree with the data in Table 1. These observations confirm that none of the autoinhibitory domain peptides is phosphorylated by CDPK.

Inhibition of CDPK Activity with Respect to ATP. The inhibitory effects of the four peptides were further examined in assays with a fixed syntide-2 concentration of 200 μM and various concentrations of ATP (5–20 μM). CDPKα 302-332 was varied from 2.5 to 10 μM. Analysis of the data by a nonlinear curve-fitting program indicated that the inhibition is mixed with respect to ATP (Figure 4A). The lines shown in the graph are the double reciprocal representation of the best-fit model. K_i and K'_i were 15 ± 2.8 and 4.5 ± 0.6 μM, respectively, for two determinations.

In contrast, inhibition of activity by the three shorter peptides with respect to ATP fit an uncompetitive model best (Figure 4 B–D). The K_i values for CDPKα 302-317, 310-332, and 318-332 were 510 ± 13 μM for two determinations, 24 ± 8 μM for three determinations, and 220 ± 13 μM for two determinations, respectively. These results confirm the ability of the peptides to inhibit CDPK activity and show that none interferes with the ATP binding site.

Formation of Peptide/Calmodulin Complexes. The autoinhibitory domains of CaMKII and MLCK contain a

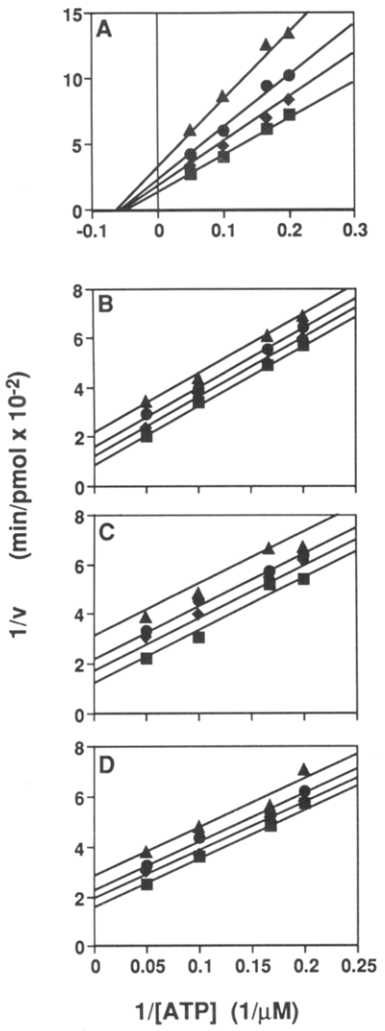

FIGURE 4: Effect of synthetic peptides on CDPK activity measured at constant syntide-2 concentration and various ATP concentrations. Activity was measured with 200 μM syntide-2 and 5, 6, 10, or 20 μM ATP. (A) CDPKα 302-332 at 0 (■), 2.5 (◆), 5 (●), and 10 (▲) μM. (B) CDPKα 302-317 at 0 (■), 200 (◆), 400 (●), and 726 (▲) μM. (C) CDPKα 310-332 at 0 (■), 10 (◆), 20 (●), and 40 (▲) μM. (D) CDPKα 318-332 at 0 (■), 50 (◆), 100 (●), and 186 (▲) μM. Error bars indicate standard deviations.

calmodulin-binding site in addition to a pseudosubstrate site. To test whether the junction domain of CDPKα also contains a binding site for calmodulin, binding of the peptides to calmodulin was examined by native gel electrophoresis. Soybean calmodulin (100 pmol, 1.7 μg) was mixed with peptides at various molar ratios (0.1, 1, and 10 mol of peptide per mol of CaM) in the presence of calcium and run on a native polyacrylamide gel (Figure 5). Soybean calmodulin, a 17-kDa acidic protein, migrated close to the dye front (lanes 1 and 20). The second, fainter, and slower migrating band observed in these lanes apparently resulted from the inclusion of calcium in the electrophoresis buffers, since only the faster migrating band was observed in gels run with EGTA (data not shown). Two peptides, CaMKII 281-309 and MLCKI, which are known to bind calmodulin, were included as positive controls. A discreet, slower migrating band was observed in lanes containing calmodulin plus CaMKII 281-309 (lanes 14–16), whereas MLCKI (lanes 17–19) caused the calmodulin band to smear. Three of the CDPKα peptides, 302-332 (lanes 2–4), 310-332 (lanes 5–7), and 318-332 (lanes 8–10), changed the mobility of the calmodulin band. In contrast, CDPKα 302-317 did not affect the mobility of the calmodulin (lanes 11–13). Control experiments showed that the peptides

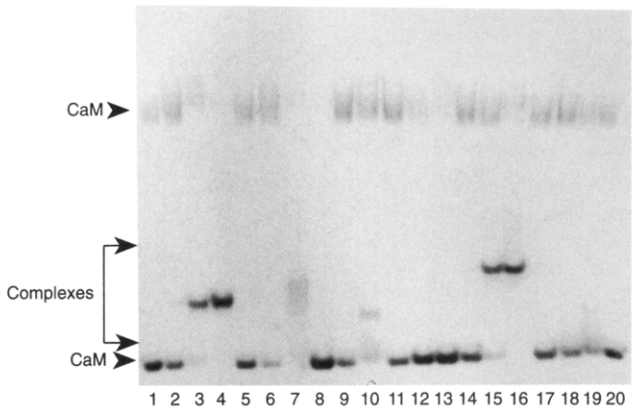

FIGURE 5: Native gel electrophoresis of calmodulin and synthetic peptides in the presence of Ca^{2+}. Soybean calmodulin (100 pmol, 1.7 μg) was electrophoresed by itself (lanes 1 and 20) or in the presence of 10, 100, or 1000 pmol of each of the following peptides: lanes 2–4, CDPKα 302-332; lanes 5–7, CDPKα 310-332; lanes 8–10 CDPKα 318-332; lanes 11–13, CDPKα 302-317; lanes 14–16, CaMKII 281-309; lanes 17–19, MLCK inhibitor peptide. $CaCl_2$ (1 mM) was added to all samples and gel buffers.

themselves, which are positively charged and predicted to migrate toward the anode, were undetectable under these electrophoresis conditions. When mixtures of peptides and calmodulin were run in native gels in the presence of 5 mM EGTA, none of the peptides caused a shift in the mobility of calmodulin (data not shown), and thus the interaction of the peptides with calmodulin was calcium-dependent. These results demonstrate directly that peptides containing residues 318-332 bind calmodulin.

To determine whether binding of the peptides to calmodulin could interfere with the function of calmodulin, their effect on the activity of skeletal muscle myosin light chain kinase (MLCK) was examined. To ensure that any observed inhibition was due to binding of the peptides to calmodulin rather that to MLCK itself, two different calmodulin concentrations were used: 2 μM, which totally saturates and fully activates the enzyme, and 20 nM, which is subsaturating and only partially activates the enzyme. As shown in Figure 6, the activity of the MLCK with 20 nM calmodulin was 20% of the activity measured at 2 μM calmodulin. In this type of experiment, inhibitor peptides that interact with the active site of the enzyme would be predicted to inhibit activity regardless of the calmodulin concentration. On the other hand, inhibitor peptides which bind to calmodulin would inhibit activity when calmodulin is limiting but not when it is in excess. MLCKI, a synthetic peptide which contains the minimal calmodulin-binding site of skeletal muscle MLCK and antagonizes calmodulin with an IC_{50} of 20.9 μM (Pearson et al., 1991), inhibited activity when calmodulin was limiting but did not inhibit activity in the presence of saturating calmodulin, provided the molar ratio of peptide to calmodulin was less than 25 (Figure 6). CDPKα 318-332 inhibited activity potently (90% inhibition at a peptide concentration of 5 μM) when calmodulin was limiting but was not able to inhibit at saturating calmodulin. In contrast, CDPKα 302-317, which does not overlap in sequence with CDPKα 318-332, inhibited activity only slightly (10% inhibition at a peptide concentration of 50 μM) when calmodulin was limiting and not at all at saturating calmodulin. Experiments with CaMKII gave similar results (data not shown). These results show that peptides containing residues 318-332 of CDPKα interfere with the binding of calmodulin to MLCK.

The sequence of the CDPKα junction domain was examined by secondary structure prediction methods. The methods of

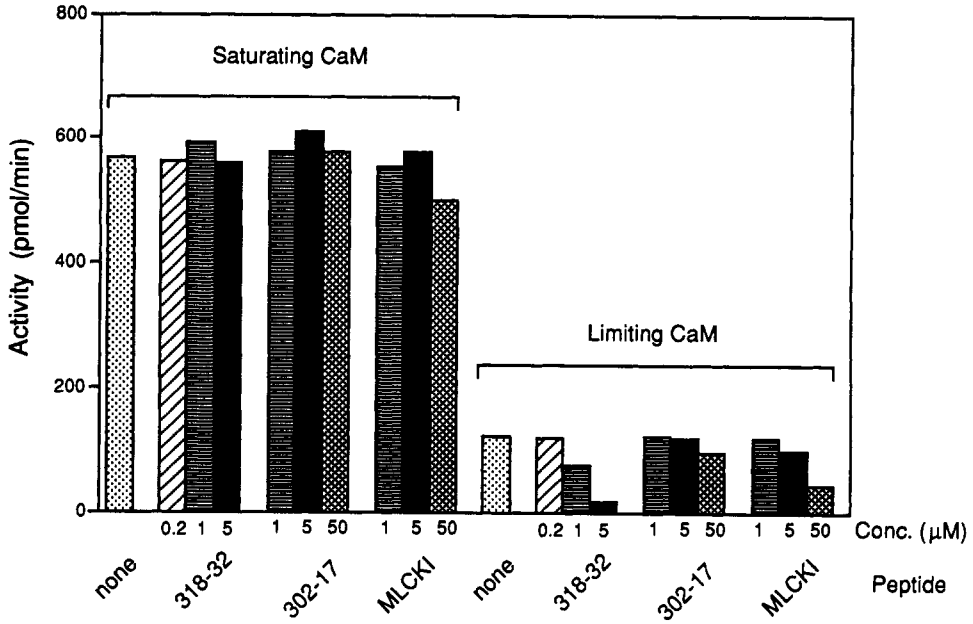

FIGURE 6: Effect of synthetic peptides on myosin light chain kinase activity. The activity of full-length skeletal muscle myosin light chain kinase was measured in the presence of saturating (2 μM) or subsaturating (20 nM) soybean calmodulin and with one of the following synthetic peptides: 0.2, 1, or 5 μM CDPKα 318-332; 1, 5, or 50 μM CDPKα 302-317; or 1, 5, or 50 μM MLCK inhibitor peptide.

	1	5	8	14
SK-MLCK M13	K R R W K K N F I A V S A A N R F K K I S S S G A L			
SM-MLCK M13	R R K W Q K T G H A V R A I G R L S S S			
Ca Pump C24W	Q I L W F R G L N R I Q T Q I R V V N A F R S S			
C20W	L R R G Q I L W F R G L N R I Q T Q I K			
Calspermin	A R R K L K A A V K A V V A S S R L G S			
Mastaporan	I N L K A L A A L A K K I L			
Mastaporan X	I N W K G I A A M A K K L L			
Melittin	G I G A V L K V L T T G L P A L I S W I K R K R Q Q			
MLCKI	K R R W K K N F I A V			
CaMPKII 290-309	L K K F N A R R K L K G A I L T T M L A			
CDPKα318-32	N K L K K M A L R V I A E R L			
CDPKα302-32	P L D S A V L S R L K Q F S A M N K L K K M A L R V I A E R L			
		310		318

FIGURE 7: Comparison of CDPKα 318-332 and 302-332 to calmodulin-binding sequences. The calmodulin-binding sequences of skeletal muscle and smooth muscle myosin light chain kinase (SK-MLCK M13 and SM-MLCK M13, respectively), calmodulin-stimulated Ca^{2+}-ATPase (Ca pump C24W and C20W), calspermin, mastaporan, mastaporan X, and melittin are aligned according to Ikura et al. (1992). Numbers above the sequences and boxes indicate residues important for binding to calmodulin. CaMKII 290-309, MLCK inhibitor peptide (MLCKI), CDPKα 318-32, and CDPKα 302-332 have been added for comparison. Numbers below the sequence of CDPKα 302-332 indicate the positions of residues 310 and 318.

Garnier–Robson (Garnier et al., 1978) and Chou–Fasman (Chou & Fasman, 1974) predict that residues 307-348 and 310-349, respectively, would form an α-helix, and the Eisenberg method (Eisenberg et al., 1984) predicts that residues 319-326 and 327-332 would form amphipathic α-helices. These predicted structures are similar to the basic amphipathic α-helix structure shown to be important for binding of certain peptides to calmodulin (O'Neil et al., 1987). Furthermore, comparison of CDPKα peptides with the calmodulin-binding motif determined by Ikura et al. (1992) (Figure 7) shows that hydrophobic residues important for interaction are present in the correct positions in two possible alignments of CDPKα 302-332. In the only alignment that is possible for CDPKα 318-332) the hydrophobic residue at position 14 in the motif is missing. This residue is also missing in Ca pump C20W and the two calmodulin-binding peptides, MLCKI and CaMKII 281-309, used in this study (Figure 7). A second alignment that is possible for CDPKα 302-332 is not possible for CDPKα 318-332. In this alignment for CDPKα 310-332, the first of the four essential hydrophobic residues is missing. Our data showing that CDPKα 318-332 but not CDPKα 302-317 binds calmodulin supports the first alignment.

Calmodulin Does Not Bind to Full-Length CDPK. The data showing that CDPKα peptides containing residues 318-332 bind calmodulin raised the question of whether calmodulin interacts with native CDPK. Previous data indicated that calmodulin has little to no effect on CDPK activity (Putnam-Evans et al., 1990), but it was still possible for calmodulin to bind to CDPK. This possibility was examined by a gel overlay technique employing biotinylated soybean calmodulin (Figure 8). In the presence of calcium, binding of biotinylated calmodulin to rabbit skeletal myosin light chain kinase (90 kDa, lane 1) but not to native (55 kDa, lane 4), recombinant full-length CDPKα (57 kDa, lane 3), or truncated CDPKα (36 kDa, lane 2) was observed. Binding of biotinylated CaM to *E. coli* proteins present in the extracts in lanes 2 and 3 was observed both in the presence (Figure 8) and in the absence of calcium (data not shown), whereas binding to MLCK was observed only in the presence of calcium. In other experiments with 50 μM biotinylated calmodulin in the overlay solution and a 2-h incubation time, Ca^{2+}-dependent binding of

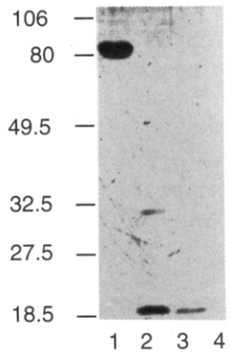

FIGURE 8: Blot overlay of CDPK and MLCK with biotinylated soybean calmodulin. Proteins resolved by SDS–polyacrylamide gel electrophoresis in the presence of 5 mM EGTA were blotted onto nitrocellulose and then overlaid with biotinylated soybean calmodulin as described in Experimental Procedures. Lane 1, 3.5 μg of skeletal muscle myosin light chain kinase; lane 2, 10 μL of extract of *E. coli* expressing CDPKα 1-312; lane 3, 10 μL of extract of *E. coli* expressing CDPKα 1-508; lane 4, 2 μg of native soybean CDPK.

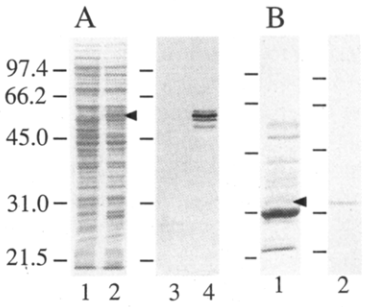

FIGURE 9: Expression of soybean CDPKα 1-508 and His_6-CDPKα 1-312 in *E. coli*. (A) Soluble extracts of *E. coli* expressing CDPKα 1-508 (lanes 2 and 4) or of control cells (lanes 1 and 3) were resolved by electrophoresis in a 10% SDS–polyacrylamide gel, transferred to nitrocellulose, and either stained with Ponceau S (lanes 1 and 2) or immunostained with monoclonal antibodies directed against soybean CDPK (lanes 3 and 4). (B) His_6-CDPKα 1-312 partially purified as described in Experimental Procedures was analyzed by SDS–polyacrylamide gel electrophoresis and stained with Coomassie Blue (lane 1) or was transferred to nitrocellulose and immunostained with monoclonal antibodies directed against soybean CDPK (lane 2). The position of the expressed protein in the gels is indicated by the arrow heads.

calmodulin to bovine brain cyclic nucleotide phosphodiesterase was observed, but again no binding to CDPK was seen (data not shown). These data show that calmodulin does not bind to CDPK bound to nitrocellulose and imply that it does not bind to CDPK in its native state.

Effect of Synthetic Peptides on the Activity of Truncated, Recombinant CDPKα. If the activity of CDPK is turned off in the absence of calcium by autoinhibition by a pseudosubstrate site, and if the activity is turned on in the presence of calcium by the binding of calcium to the regulatory domain, then it would be predicted that removal of the pseudosubstrate site and the calcium-binding domain would yield an enzyme having activity in both the presence and the absence of calcium. To test this prediction, we expressed full-length and truncated forms of CDPKα in *E. coli*. Most of the expressed protein was in inclusion bodies, but some was present in soluble extracts (Figure 9). Activity in extracts containing full-length CDPKα (residues 1-508) was stimulated 50–100-fold by micromolar free calcium. Negligible phosphorylation of syntide-2 was observed when extracts of untransformed or mock-transformed *E. coli* were used. The activity of enzyme truncated at residue 296 (His_6-CDPKα 1-296), containing the catalytic domain and no residues from the autoinhibitory or calcium-binding domain, was labile during dialysis, and thus active enzyme could not be purified. However, His_6-CDPKα 1-312, containing the catalytic domain plus 16 residues from the junction domain and six added histidine residues at its amino terminus, was stable and was partially purified by chelation chromatography and ion-exchange chromatography. Analysis of the enzyme by SDS–PAGE and immunoblotting shows that the enzyme is a minor component of the preparation (Figure 9). Numerous protein bands are seen in the Coomassie Blue-stained gel, but only a single immunodecorated band corresponding to a molecular weight of 33 000 is seen in the immunoblot. The immunodecorated band does not correspond to the most heavily stained protein band, which has a molecular weight of 31 000. His_6-CDPKα 1-312 was active in the absence of calcium (55 nmol/min/mg) and was slightly stimulated (66 nmol/min/mg) by micromolar free calcium. Similar results were obtained with CDPKα 1-312, which did not have His_6 added to its amino terminus. These values were 2 orders of magnitude lower than those for CDPK purified from soybean cells and reflect the impurity of the preparation but may also be a result of the presence of some of the residues from the junction domain. It is evident, however, that the activity of this truncated enzyme is only slightly affected by calcium and that the carboxyl-terminal 196 residues are required for regulation by calcium.

Synthetic peptides that inhibit native CDPK by interaction with the catalytic domain should also inhibit His_6-CDPKα 1-312. CDPKα 318-332 was a poor inhibitor and had an IC_{50} of 800 μM, whereas CDPKα 310-332 inhibited activity, with an IC_{50} of 30 μM (Figure 10A). Inhibition by CDPKα 310-332 was competitive with syntide-2 (Figure 10B). The K_i values for two determinations were 4.1 and 5.4 μM. These results show that the eight amino acids, 310-317, present in this peptide but missing from CDPKα 318-332 contribute significantly to inhibition of activity. The longest peptide, CDPKα 302-332, potently inhibited the catalytic domain, with an IC_{50} of 4 μM, but inhibition was mixed, suggesting that it binds to a site remote from the active site. CDPKα 302-317 inhibited the activity of His_6-CDPKα 1-312, with an IC_{50} >100 μM. However, this peptide inhibited the ΔJC construct of *Arabidopsis* AK1, described by Harper et al. (1994) with an IC_{50} of 90 μM (data not shown). The ΔJC construct contains no residues of the autoinhibitory domain.

DISCUSSION

Evidence reported here supports the hypothesis that CDPK is regulated by an autoinhibitory domain located in the junction between the catalytic and calmodulin-like domains. Synthetic peptides corresponding to the junction domain inhibit the activity of native soybean CDPK (Figures 2 and 4) with K_i values as low as 1.9 μM (Table 2). Three peptides, CDPKα 310-332, 302-317, and 318-332, inhibited the activity of the native enzyme competitively with respect to syntide-2 (Figure 2). The latter two peptides were 5- and 17-fold less potent, respectively, than CDPKα 310-332 (Table 2). Since CDPKα 310-332 contains all of the residues in CDPKα 318-332 and eight residues from CDPKα 302-317, it is reasonable to propose that residues in each of the smaller peptides contribute to the more potent inhibitory site contained in CDPKα 310-332.

Competitive inhibition implies that the peptides are interacting with the active site of the enzyme. But since CDPKα 310-332 binds to calmodulin (Figure 5), it is also possible that this peptide inhibits native CDPK by binding to its calmodulin-like domain. Inhibition would be competitive with syntide-2 if the binding of the peptide to the calmodulin-like domain indirectly interfered with the binding of syntide-2. To

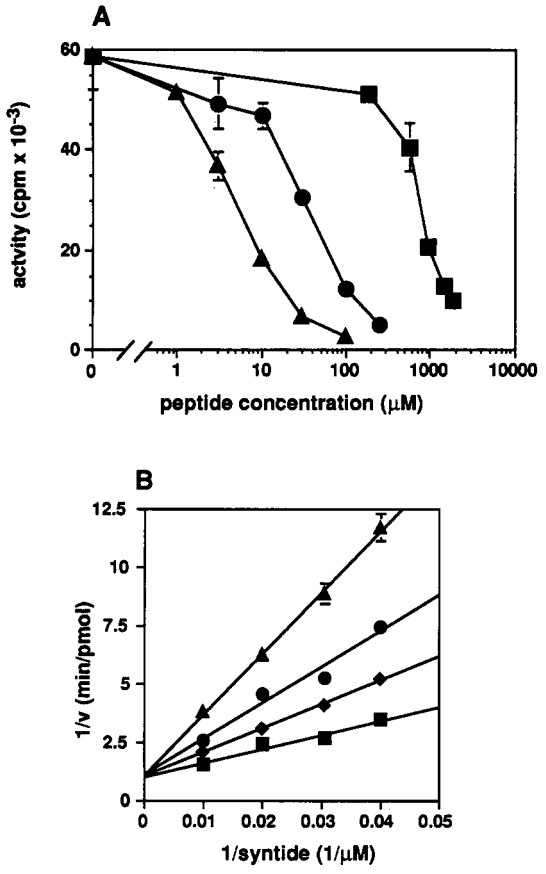

FIGURE 10: Inhibition of His_6-CDPKα 1-312 by synthetic peptides. (A) The activity of His_6-CDPKα 1-312 was determined with 200 μM syntide-2 and 60 μM ATP and various concentrations of CDPKα 302-332 (▲), CDPKα 310-332 (●), or CDPKα 318-332 (■). (B) Kinetic analysis of the inhibition of His_6-CDPKα 1-312 by CDPKα 310-332 was determined as described in the legend to Figure 2. The concentrations of CDPKα 310-332 were 0 (■), 5 (◆), 10 (●), and 40 (▲) μM.

investigate this possibility, we engineered a recombinant CDPK, His_6-CDPKα 1-312, which contains the catalytic domain and a few residues of the junction domain but none of the calmodulin-like domain. This partially purified enzyme has a low specific activity relative to the native CDPK, but its activity was unaffected by calcium. CDPKα 310-332 inhibited the activity of the truncated CDPK competitively with respect to syntide-2 and the K_i (4.1, 5.4 μM for two determinations) was similar to that determined for native CDPK (4.8 μM). These data clearly show that peptide CDPKα 310-332 inhibits the enzyme by interaction with the catalytic domain at or near the active site. Thus a site between residues 310 and 332 is capable of acting as a pseudosubstrate sequence. The mutational analysis of the *Arabidopsis* CDPK, AK1 reported by Harper et al. (1994), is consistent with this conclusion.

The longest peptide tested, CDPKα 302-332, inhibited activity by a mixed mechanism. This observation shows that this peptide binds to a site other than the active site or possibly to ATP. Since neither CDPKα 302-317 nor CDPKα 318-332 inhibited with mixed kinetics, it is evident that residues at both ends of 302-332 are required for interaction with the other site. Whether this interaction is important for regulation of the holoenzyme is not known.

Although peptides containing basic-X-X-Ser motifs were good substrates of CDPK [Table 1 and Roberts and Harmon (1992)], three CDPK peptides, CDPKα 302-332, 302-317, and 310-332, containing the sequence KQFS were not phosphorylated. This observation shows that residues outside of the motif may influence the ability of the enzyme to phosphorylate the sequence. Furthermore, these results suggest that this KQFS is not a site of autophosphorylation in CDPK, but this suggestion must be examined through direct determination of the site(s) of autophosphorylation.

The KQFS sequence could contribute to the pseudosubstrate site, however, since addition of residues 310-317 to peptide 318-332 reduced the K_i from 25 to 5 μM (Table 2). Comparison of the sequences CDPKα 310-332 and the pseudosubstrate sequence of CaMKII (residues 290-309) shows that they have eight identical residues (Figure 1). These peptides are competitive inhibitors of their respective enzymes (Payne et al., 1988; Smith et al., 1992). These similarities further suggest that residues in CDPKα 310-332 act as a pseudosubstrate site.

Earlier kinetic analysis of CDPK showed that ADP is a competitive inhibitor with respect to ATP (K_i = 18 μM) and suggested that CDPK's reaction mechanism is sequential (Putnam-Evans et al., 1990). These results, taken together with our current finding that three peptides are competitive inhibitors with respect to syntide-2 but uncompetitive inhibitors with respect to ATP (Table 2), suggest that the binding of substrates to the enzyme is ordered, with ATP binding first. A similar binding order was found for CaMKII (Kwiatkowski et al., 1990) and for cAMP-dependent protein kinase, which has an ordered bi–bi mechanism (Whitehouse et al., 1983).

The autoinhibitory domain of CaMKII interferes with the ATP-binding site as well as with the peptide-binding site (Colbran et al., 1989b; Payne et al., 1988; Smith et al., 1992). In contrast, none of the CDPK peptides tested was competitive with ATP. Our results showing uncompetitive inhibition with respect to ATP by synthetic peptides that compete with syntide-2 (Table 2) differ from those of similar studies with CaMKII. In those studies, peptides corresponding to the pseudosubstrate site, CaMKII 290-302 and 290-309, were partially competitive with syntide-2, with IC_{50} values of 53 and 24 μM, respectively (Payne et al., 1988). The partial inhibition by the peptides indicates that they are not deadend inhibitors of CaMKII and explains why inhibition with respect to ATP by these peptides is noncompetitive (Smith et al., 1992) rather than uncompetitive, as predicted by the ordered binding of ATP and syntide observed in other studies.

Although three peptides, CDPKα 302-332, 310-332, and 318-332, bind to soybean calmodulin, biotinylated calmodulin did not bind to CDPK in a blot overlay assay. CDPK blotted onto nitrocellulose is able to renature, at least partially, since it retains activity (Verhey et al., 1993). We interpret our results to mean that calmodulin does not bind to CDPK in its native state. It is possible that when the enzyme is in its active state, residues 318-332 are inaccessible to exogenous calmodulin.

The observation that CDPKα peptides 302-332, 310-332, and 318-332 bind to calmodulin is significant for three reasons. Firstly, this result shows that these peptides are not specific inhibitors of CDPK and would not be useful in studies in vivo or in studies employing crude extracts. Secondly, the presence of a calmodulin-binding site between residues 318 and 332, as well as a pseudosubstrate sequence between residues 310-332, in the junction domain of CDPK demonstrates that this domain and the autoinhibitory domain of CaMPKII have similar functional capabilities. However, our results showing that calmodulin does not bind to the full-length CDPK (Figure 8) suggest that calmodulin is not important in the regulation of CDPK in vivo. Thirdly, since the calmodulin-like domains

of CDPK and calmodulin have some similarity in their binding properties (Harmon et al., 1987; Putnam-Evans et al., 1990; Roberts & Harmon, 1992), this observation suggests that the calmodulin-like domain is capable of binding to the peptides and possibly to the autoinhibitory domain of the enzyme.

We have shown that sequences in the autoinhibitory domain of CDPK may act as a pseudosubstrate site that turns activity off. Demonstration that sequences within the autoinhibitory domain bind to calmodulin opens the interesting possibility that autoinhibition of CDPK might be relieved in the presence of calcium by a mechanism that is analogous to that of the calmodulin-dependent protein kinases. Both myosin light chain kinase and calmodulin-dependent protein kinase type II are regulated by the binding of calmodulin to their autoinhibitory domains (Bagchi et al., 1992; Kemp & Pearson, 1991; Soderling, 1990). As applied to CDPK, the model would involve the binding of the calmodulin-like domain to the autoinhibitory domain. This possibility is currently under investigation.

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