Genetic Identification of an Autoinhibitor in CDPK, a Protein Kinase with a Calmodulin-like Domain†

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ABSTRACT: CDPKs are a family of calcium (Ca^{2+})-dependent protein kinases which are defined by a carboxyl-terminal calmodulin-like domain. Mutational analysis indicates that the junction domain, which joins the kinase and calmodulin-like domains, contains an autoinhibitor. CDPK isoform AK1 from *Arabidopsis* was expressed in *Escherichia coli* as a fusion protein sandwiched between glutathione S-transferase and six consecutive histidines at the N- and C-terminal ends, respectively. This fusion, called AK1-6H, was purified and displayed kinase activity which was stimulated up to 127-fold by Ca^{2+}, with a typical specific activity of 2000 nmol min^{-1} mg^{-1}, using syntide-2 as peptide substrate. A truncation which deletes the calmodulin-like domain, as in mutant ΔC-6H, disrupts Ca^{2+} activation and leaves the enzyme with a basal level of activity. ΔC-6H could be activated 87-fold by preincubation with a purified polyclonal IgG which was raised against a junction domain fusion. A further deletion of the junction domain, as in mutant ΔJC, results in a constitutively active enzyme. This indicates that the junction domain in ΔC-6H can function as an autoinhibitor. Its function as an autoinhibitor in a full-length enzyme was confirmed by site-specific mutagenesis, as shown by mutant KJM23-6H, which had a six-residue substitution in the junction domain between A_{422} and A_{432}. Both ΔJC and KJM23-6H encoded Ca^{2+}-independent enzymes which had specific activities greater than 70% that of a fully active AK1-6H and displayed equivalent K_m values for ATP and syntide-2. Inhibition studies on ΔJC, using peptides based on the autoinhibitory domains of Ca^{2+}/calmodulin-dependent protein kinases, are consistent with a model where the junction domain contains a similar pseudosubstrate-type autoinhibitor.

CDPKs[1] represent a third family of calcium (Ca^{2+})-regulated protein kinases [Harper et al., 1991; reviewed in Roberts and Harmon (1992); Roberts (1993); and Poovaiah and Reddy (1993)]. They are biochemically distinct from protein kinase C and Ca^{2+}/calmodulin-dependent protein kinases since they do not require either lipid or calmodulin for activation. Their unique biochemistry appears to result from a novel structural arrangement. Within a single polypeptide, a kinase domain is joined to a calmodulin-like regulatory domain which contains four Ca^{2+}-binding EF-hands. Although the physiological functions of CDPKs are not known, their unusual structure may provide the basis for a novel Ca^{2+}-mediated signal transduction pathway.

CDPKs were first discovered in plants (Harmon et al., 1987; Harper et al., 1991) but have also been found in protozoa (Zhao et al., 1993). The prototype CDPKα was cloned from soybean, based on peptide sequence from a purified enzyme (Harper et al., 1991). Isoform AK1, which is used in this investigation, was cloned from a model higher plant, *Arabidopsis*, and is distinguished from CDPKα by the presence of a unique 116-residue N-terminal domain (Harper et al., 1993). Isoform AK1 was expressed in *Escherichia coli* and shown to encode a Ca^{2+}-dependent protein kinase. Research on the *Arabidopsis* and soybean genomes indicates that CDPKs are encoded by a large multigene family (Harper et al., 1991, 1993). CDPK genes have also been cloned from carrot (Suen & Choi, 1991), rice (Kawasaki et al., 1993), corn (Poovaiah & Reddy, 1993), and the protist *Plasmodium*, a malarial parasite (Zhao et al., 1993). In *Paramecium*, two CDPK-like enzymes have been purified to homogeneity (Son et al., 1993), which suggests that this enzyme family is widespread in protozoa.

In a comparison of CDPKs from plants, one of the most highly conserved regions is a 31 amino acid sequence which joins the kinase and calmodulin-like domains together (Harper et al., 1993). This region was called the junction domain because of its obvious structural function. In addition, it was proposed to function as an autoinhibitor (Harper, et al., 1991, 1993; Roberts & Harmon, 1992), based on analogy to Ca^{2+}/calmodulin-regulated protein kinases, such as Ca^{2+}/calmodulin-dependent protein kinases II (CaMKII) and myosin light chain kinase (MLCK), in which this position is occupied by a pseudosubstrate-type autoinhibitor (Kemp & Pearson, 1991).

CaMKIIβ was chosen as a paradigm for investigating the regulatory mechanism of CDPK since it was most closely related to CDPK on the basis of amino acid sequence identity in the kinase catalytic core (CaMKIIβ shares 39% identity with CDPKα and AK1; Harper et al., 1991, 1993). The activity of CaMKIIβ appears to be maintained at a low basal level by a pseudosubstrate-type autoinhibitor (Hanson & Schulman, 1992; Kemp & Pearson, 1991). Activation occurs when Ca^{2+}/calmodulin binds to a region which adjoins or overlaps with the autoinhibitor. Other Ca^{2+}/calmodulin-regulated kinases, such as MLCK, have analogous regulatory mechanisms (Kemp & Pearson, 1991; Kemp et al., 1991). Nevertheless, CDPKs are distinct from calmodulin-regulated

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[1] Abbreviations used: CDPK, calcium-dependent (or calmodulin-like domain) protein kinase; GST, glutathione S-transferase; 6×His, six consecutive histidines; AK1, *Arabidopsis* kinase-1: AK1-6H, AK1 fusion protein sandwiched between a GST at the N-terminus and a 6×His motif at the C-terminus; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; IC_{50}, concentration of a molecule resulting in 50% inhibition of enzyme activity under standard assay conditions.

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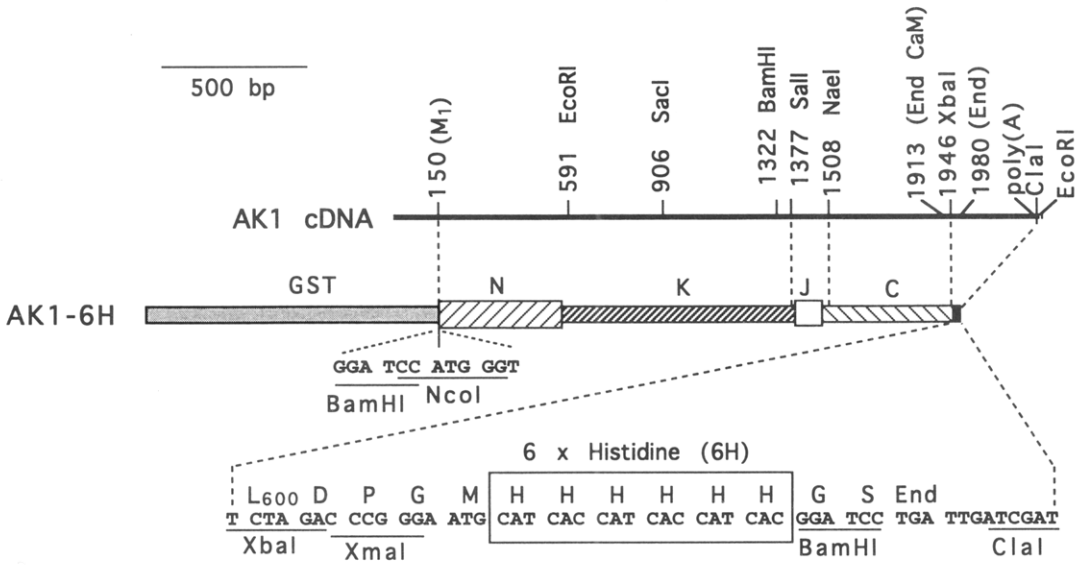

FIGURE 1: Diagram of AK1-6H. The cDNA sequence of AK1 (GeneBank accession number L14771) is diagrammed at the top. Important positions in the coding sequence are indicated by base pair number. The coding sequence extends from the start methionine (M_1) at 150 to a stop codon at 1980. The calmodulin-like domain ends at position 1913. Restriction sites used in constructions are indicated. The affinity sandwich fusion AK1-6H is shown below, with dotted lines indicating the relationship to the cDNA. The glutathione S-transferase domain (GST) is followed by a 15-kD N-terminal domain (N), a kinase catalytic core (K), a junction domain (J), a calmodulin-like domain (C), and six consecutive histidines (6H). The 6×His motif is enlarged at the bottom to show the nucleotide and amino acid sequence. The vector used was pGEX-2T (Smith & Johnson, 1988). The AK1 insert begins at a *Bam*HI site and ends after a 6×His motif at an *Eco*RI site. (Note: the *Bam*HI and *Eco*RI sites are not unique.)

kinases since a calmodulin-like domain is permanently attached. This novel structure raises alternative mechanisms of regulation which depart from those of a stereotypical Ca^{2+}/calmodulin-regulated protein kinase.

The purpose of this investigation was to distinguish between two general models for CDPK autoinhibition. The first model proposes that an autoinhibitor is located immediately C-terminal of the kinase domain, consistent with a structural analogy to Ca^{2+}/calmodulin-dependent protein kinases. In the second model, autoinhibition is proposed to be an intrinsic feature of the kinase catalytic core. This model is based on analogy to CDC2 kinase (De Bondt et al., 1993), which appears to be activated by an allosteric change in the structure of the catalytic core, induced by the binding of cyclin at a distant location. Similarly, CDPK may be activated by an allosteric change in the kinase domain structure as a result of a conformational change initiated in the calmodulin-like domain. In this model, an explanation for the conserved sequence found in the junction is that it provides an important conduit for the transduction of a conformational change.

Our investigation is presented in three parts. First, we show that an AK1 fusion protein expressed in *E. coli* represents a valid model for investigating Ca^{2+} activation of CDPKs. AK1 was expressed as an affinity sandwich fusion protein called AK1-6H. This affinity sandwich design permitted full-length protein to be purified free of contaminating N- or C-terminal truncation products. Second, we describe our use of mutational analysis to identify the junction domain as the location of an autoinhibitor in CDPKs. This position is analogous to the position of autoinhibitors in Ca^{2+}/calmodulin-dependent protein kinases. Finally, we show that a pseudosubstrate peptide inhibitor of MLCK can also function as a pseudosubstrate inhibitor of AK1. This suggests that the two kinases may utilize a similar mechanism of autoinhibition. Peptide inhibitor studies (Harmon et al., 1994) confirm that the junction domain contains a pseudosubstrate autoinhibitor.

MATERIALS AND METHODS

The *E. coli* hosts used for cloning and production of fusion proteins were DH10α and XL-1 Blue (Strategene, La Jolla, CA). DNA template for oligonucleotide site-directed mutagenesis was produced in strain CJ236 (Kunkel et al., 1987). Unless otherwise mentioned, all standard molecular techniques were performed according to Maniatas et al. (1982).

[γ-^{32}P]ATP was obtained from Amersham (Arlington Heights, IL). Peptide substrate syntide-2 (sequence PLARTLSVAGLPKK) was a gift from A. Harmon (University of Florida, Gainesville, FL) and later obtained from Bachem (Torrance, CA). Glutathione Sepharose 4B was obtained from Pharmacia (Piscataway, NJ), and nickel resin (ProBond) was from Invitrogen (San Diego, CA). Crude lipid (phosphatidylcholine) was obtained from Sigma (St. Louis, MO).

AK1 Fusion Construct. AK1-6H contains the coding sequence for isoform AK1 sandwiched between a glutathione S-transferase (GST) and six consecutive histidines (6×His) (Figure 1). AK1-6H is based on construct Com-7, which was previously described (Harper et al., 1993) and was created by the addition of a 6×His motif to the C-terminal end.

Truncation Mutants. N- and C-terminal truncations are diagrammed in Figure 3.

ΔN-6H is a truncation of AK1's N-terminal 124 amino acids with the GST fused to residue M_{125}. ΔN-6H was constructed by adding the C-terminal half of AK1-6H [from *Sac*I (base pair position 906) to *Pst*I (vector site distal to end of coding sequence)] to the N-terminal portion of KGF-1 (Harper et al., 1993).

ΔC-6H (identified as clone ΔC-6H1 mp#4) is a deletion of the calmodulin-like domain from AK1-6H but with retention of the 6×His motif at the C-terminus. ΔC-6H was constructed by digesting AK1-6H with *Xba*I and *Nae*I, filling sticky ends with Klenow, and blunt end ligation. The first 10 amino acids of the calmodulin-like domain are present (A_{445} to A_{454}), followed by a 6xHis motif.

ΔJC is a truncation of AK1-6H which removes the junction and calmodulin-like domains. Two independent constructs, ΔJC-2 and KJM26-ΔJC, were made because of the potential for negative selection of a less active enzyme during cloning. However, both cloning pedigrees gave equivalent enzyme and are called ΔJC in the text. For clone ΔJC-2, the parent clone was Com-7, which was truncated by cutting with *Sal*I and *Xba*I, Klenow filling, and blunt end ligation. The DNA sequence of this fusion site is gtc/gac/*tag*. This ligation recreated a *Sal*I site (underlined) and provided an in-frame TAG stop codon. This sequence was verified by DNA sequencing. The predicted protein sequence ends with residue D_{411}. In the second clone, KJM26-ΔJC, an identical truncation was made, but it came from a different parent, KJM26-6H6, which had a long history of subcloning (see below).

ΔJC-6H3 is also a deletion of the junction and calmodulin-like domains, but instead of an immediate translational termination after residue D_{411}, as in ΔJC above, this construct has a 21 amino acid extension containing a Factor Xa protease cleavage site and ending with a 6×His motif. This construct was made from AK1-6H by replacement of sequence between the *Sal*I site (preceding the junction domain) and the *Xma*I site (immediately preceding the 6×His motif) with an oligo SB1+2 (taa/gtc/gac/ggt/atc/gag/gga/agg/ata/gat/ctc/gag/tct/ccg/gaa/at; the *Sal*I and *Bsp*EI sites are underlined). Two similar constructs were made with a 6×His motif included at the end of slightly shorter extensions. ΔJC6H1 has a 10 amino acid extension (with D_{411} mutated to E), which was made by cutting AK1-6H with *Sal*I and *Sma*I, Klenow filling, and blunt end ligation. ΔJC6H4 has a 17 amino acid extension, which was made by cutting AK1-6H with *Sal*I and *Xba*I and replacing that region with a *Sal*I/*Xba*I adapter (SX1+2) with the sequence gtc/gac/ggt/gga/gg a/gat/cta/ga (a *Bgl*II site is underlined). The new C-terminal end has the predicted sequence of VD_{411}GGGDL (6×His motif).

ΔJC(26)-6H is also a deletion of the junction and calmodulin-like domains but has a longer 61 amino acid extension (starting at D_{411}), which includes a terminal 6×His motif. This clone was made from KJM26-6H6 (see below) by cutting with *Xma*I and religation.

Junction Mutants. Mutations within the junction domain are diagrammed in Figure 6.

KJM23-6H2 has a mutation of six amino acids in the junction domain. The mutations were engineered by site-specific mutagenesis according to a published procedure (Kunkel et al., 1987; kit from Bio-Rad, Hercules, CA) using ssDNA template from clone pAK-NX-25 (Harper et al., 1993) and the oligo KJ1 (tg/gat/tct/ccg/gat/ctg/agc/cgt/atg/aat/ccg/gaa/gat/cta/atg/aac/a; two *Bsp*EI sites and a *Bgl*II site are underlined). The original mutant clone was called KJM-1. KJM-23-6H2 was made by a direct replacement of the *Sal*I/*Xba*I fragment in clone AK1-6H with that from mutant KJM-1.

KJM25-6H2 is a replacement of 11 amino acids in the junction domain (A422 to A432) with a c-myc epitope (oligo c-mycI/II, aag/gat/cct/ccg/gag/caa/aag/ctt/atc/agt/gat/gaa/gat/ctc/ccg/gga/tta/a; *Bam*HI, *Bsp*EI, *Hin*dIII, *Bgl*II, and *Xma*I sites are underlined). This clone was made from KJM23-6H2 by inserting a *Bsp*EI and *Bgl*II fragment encoding a c-myc epitope. The c-myc epitope (Munro & Pelham, 1986) was chosen as an inert replacement sequence because of its potential usefulness in immuno-detection, using commercially available monoclonal antibodies (Oncogene Science, Manhasset, NY) in future *in vivo* expression experiments.

KJM26-6H4 is a replacement of the 31 amino acid junction domain with a 30 amino acid sequence containing two Factor Xa sites and a c-myc epitope. KJM26-6H6 is similar but has a larger substitution (52 amino acids) containing three Factor Xa sites and two c-myc epitopes. The replacements were made between the *Sal*I site on the 5′ side of the junction domain and an *Xma*I site engineered into the 3′ side of the junction domain using oligo KJ2 (g/aaa/atg/gca/gat/ctc/ccg/ggt/gct/gag/agc/tta; *Bgl*II and *Xma*I sites are underlined; the final codon corresponds to L448). The cloning pedigree of KJM26-6H6 and -6H4 is complicated and will be only briefly outlined here. KJM-1 and KJM-2 are the original clones engineered with mutations encoded by oligos KJ1 and KJ2, respectively. Fragments (*Sac*I/*Xba*I) from these clones were gel purified and subcloned into Com-7 to give rise to KJM3 and KJM4, respectively. KJM6 was generated by fusing KJM3 and KJM4 at their respective *Bgl*II sites located in their junction domains. KJM8 was generated from KJM6 by insertion of a single c-myc I/II epitope (encoded in a *Bam*HI/*Xma*I fragment) into the *Bgl*II/*Xma*I site. KJM8-6H has 6×His motif at the C-terminus and was made by replacing the junction and calmodulin-like domains in AK1-6H with the *Sal*I/*Xba*I fragment from KJM8. KJM26-6H2 was generated by combining KJM8-6H and KJM12 at a *Bsp*EI site such that the calmodulin-like domain and the 6×His motif were from KJM8-6H and the kinase and N-terminal domains were from KJM12. KJM12 was from an independent pedigree and was used here to obtain a single Factor Xa site in the correct location. The Factor Xa site and c-myc sequence were duplicated to make construct KJM26-6H3 by removing the *Sal*I/*Xma*I fragment from KJM26-6H2 and reinserting it into a KJM26-6H2 cut with *Xho*I/*Bsp*EI. KJM26-6H4 was derived from KJM26-6H2 by the addition of second Factor Xa site encoded in a *Sal*I/*Bsp*EI fragment from oligo SB1+2. Finally, KJM26-6H6 was generated by cutting KJM26-6H3 with *Xho*I and *Bsp*EI and adding a third Factor Xa site using oligo SB1+2 cut with *Sal*I and *Bsp*EI. To verify that the kinase domain which was present in the final KJM26-6H6 construct was still fully active, KJM26-6H6 was converted to a ΔJC and shown to have the same activity as an identical ΔJC which was cloned via a more direct route.

KJM13-6H is identical to AK1-6H except that it has an insertion of a Factor Xa site immediately preceding the junction domain. The SB1+2 oligo encoding a Factor Xa site was cloned as a *Sal*I/*Xho*I fragment into the *Sal*I site of Com-7. A 6×His motif from AK1-6H was subsequently added to the *Xba*I site at the C-terminal end.

J-6H/KG is a GST fusion containing the junction domain. It was constructed by taking the sequence encoding the 57 C-terminal residues of ΔC-6H and inserting it into vector pGEX-KG (Guan & Dixon, 1991). The fusion site is the *Sal*I site immediately preceding the junction domain and contains residues D_{411} to A_{454} (relative to AK1) followed by a 6×His motif.

DNA Sequencing. Site-specific mutations were confirmed by DNA sequencing or by restriction analysis when subcloned into a new construct. DNA sequencing was performed using a Sequenase Kit (U.S. Biochemicals) or on an Applied Biosystems automated DNA sequencer operated by the Scripps Biotechnology Core Facility.

Affinity Sandwich Purification. A general protocol is presented which was used to purify full-length fusion proteins sandwiched between a GST domain and 6×His motif. This protocol differs from that described by Binder et al. (1994)

and appears to yield enzyme of higher specific activity. *E. coli* (DH10α) harboring recombinant plasmids were grown under ampicillin selection in nutrient broth 2×YT. Overnight cultures grown at 37 °C were diluted 10-fold and grown for 3 h at 30 °C before isopropyl β-D-thiogalactopyranoside (IPTG) was added to 0.5 mM and growth was continued for an additional 1.5 h. All subsequent purification steps were carried out at 4 °C. A 220-mL aliquot of culture was centrifuged for 10 min at 5000*g*, and the pellet was resuspended in 10 mL of lysis buffer containing 20 mM Tris-HCl (pH 7.8), 500 mM NaCl, 14 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were lysed by the addition of lysozyme to 1–2 mg mL^{-1}, incubation on ice for 15 min, and addition of Triton X-100 to 0.4%, followed by sonication. In some cases, lysozyme was omitted and cells were lysed by sonication or a French Press; no significant difference was observed for kinase activities for enzymes prepared by different lysis methods. Cellular debris and unlysed cells were removed by centrifugation at 12 000*g* for 10 min. The cleared supernatant was incubated for 30 min with approximately 250 μL of nickel resin (ProBond) on a rocking platform. Resin was pelleted in a table-top centrifuge and washed extensively with 20 mM Tris-HCl (pH 7.8) and 500 mM NaCl and eluted with 300 mM imidazole in 20 mM $NaPO_4$ (pH 6.0). The eluate was diluted 5-fold with GST binding buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM EDTA, 1 mM dithiothreitol (DTT), and 0.4% Triton X-100) and incubated with approximately 200 μL of glutathione Sepharose 4B for 30 min. The beads were washed extensively with binding buffer, followed by two washes with 50 mM Tris (pH 7.5). Protein was eluted with 5–10 mM glutathione in 50 mM Tris-HCl (pH 8.0) and concentrated by centrifugation in a Centricon-30 tube (Amicon, Beverly, MA). Enzyme was stored at –70 °C (long term) or –20 °C (short term) in 50% glycerol, 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, and 1 mM DTT. A couple of freeze–thaw cycles did not significantly affect the activity of AK1-6H. Although this outline describes affinity chromatography steps performed by a batch method, a column method was found to be equally effective and was used for many of the enzyme preparations. Typically, a preparation starting with 220 mL of culture yielded approximately 0.1–0.2 mg of pure AK1-6H.

Q-Sepharose Purifications. Since ΔJC did not contain a C-terminal 6×His motif, it was further purified by Q-Sepharose ion-exchange chromatography following a single GST affinity chromatography step. Protein preparations started with 0.5–1.2 L of culture. GST-purified protein was loaded onto a Bio-Rad Poly-Prep chromatography column with a 1-mL bed volume of Q-Sepharose equilibrated with 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0.1 mM EDTA. The column was washed with 10 mL of equilibration buffer. Bound protein was eluted in 2-mL fractions with 20 mM Tris (pH 7.5), 0.1 mM EDTA, and increasing NaCl concentrations of 200, 300, 400, and 500 mM. Protein was concentrated in a Centricon-30 tube and brought to a final concentration of 50% glycerol, 0.1 M NaCl, and 1 mM DTT for storage.

Expression in E. coli. Constructs with unregulated kinase activity, such as ΔJC, grew very poorly in host strains XL1-Blue and DH10α. One interpretation is that leaky expression of an unregulated kinase is toxic. Therefore, care was taken during this study to minimize the chance for selection of mutants with reduced activity. For example, in protein production, cultures were seeded directly from the original stock kept frozen at –70 °C in 20% glycerol. An alternative host, BL-21(DES-3), was evaluated since it was reported to have a reduced level of leaky expression. This host was rejected after a high frequency of DNA rearrangements was observed for two independent clones. Alternative *E. coli* hosts were also examined as a way to increase yield of pure enzyme and reduce the level of unwanted truncation products. Several *lon*–protease-deficient strains were tested with no apparent benefits.

One caveat of any fusion protein system is that an affinity tag may change the kinetic properties of the enzyme. Since a native AK1 enzyme has not been purified from *Arabidopsis*, a direct comparison cannot be made. Nevertheless, two N-terminal affinity tags were tested for their influence on kinase activity. AK1 fusions were made with either a GST (i.e., AK1-6H) or a maltose binding protein (ComC-6H, vector pMALcR1; data not shown). Each enzyme displayed equivalent kinase activity after affinity sandwich purifications. The maltose binding protein system (New England Biolabs, Beverly, MA) may offer an advantage over GST fusions since cleaner fusion protein was obtained after a single affinity selection step.

Kinase Assay. The protein kinase activities of fusion proteins were assayed in 20 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 1 mM EGTA, 0.5 mg mL^{-1} bovine serum albumin (ultrapure, fatty acid-free), 100 μM syntide-2, 300 μM ATP, and 50 ng of purified enzyme in a 60-μL reaction. For Ca^{2+} stimulation, assays included 1.1 mM $CaCl_2$ to provide an excess of free Ca^{2+} to levels greater than 100 μM. Unless noted, our standard assay included crude lipid (phosphatidylcholine) at 1 mg mL^{-1}. Crude lipid mixtures were suspended in buffer by probe sonication. Reactions were quantitated using 1–10 μCi [γ-^{32}P]ATP per reaction. Assays were initiated by the addition of ATP and transferred from ice to room temperature (20–22 °C) for a 15-min incubation. Reactions were terminated by spotting 25 μL of the assay onto phosphocellulose filter paper (P81) from Whatman (Casnellie, 1991) and immersing it into 75 mM phosphoric acid (10 mL per reaction). Filters were washed with four 5-min changes of the same solution. Control experiments indicated that the phosphorylated syntide-2 strongly adhered to the paper even in the presence of crude lipid (B. Binder, personal communication). Total radioactive ATP per reaction was determined by transferring a sample of the reaction mixture to P81 paper and drying without washes. Radioactivity was measured by Cerenkov radiation in a Beckman LS 3801 scintillation counter.

Gel Analysis and Western Blots. Proteins were separated by SDS–PAGE (Laemmli, 1970) and transferred to nitrocellulose in a Bio-Rad Mini Trans-Blot electrophoretic transfer cell using 25 mM Tris-HCl (pH 8.6), 192 mM glycine, 0.2% (w/v) SDS, and 20% (v/v) methanol. Western blots were blocked with a 5% (w/v) nonfat powdered milk solution in 10 mM Tris (pH 8.0), 150 mM NaCl, and 0.5% Tween-20. Blots were incubated with an anti-CDPK monoclonal antibody (14G5, gift from A. Harmon) at a concentration of 1 μg mL^{-1} (i.e., 2000× dilution) in the same blocking buffer with 0.1% (w/v) milk for 1 h at room temperature. Immunodecorated proteins were visualized with an anti-mouse secondary antibody conjugated to alkaline phosphatase (Promega, Madison WI). The anti-CDPK antibody recognizes an epitope in subdomain 3 of the kinase domain of many CDPKs (A. Harmon, personal communication).

Antibody Purification. Rabbit polyclonal antiserum (T857) was raised against fusion protein J-6H/KG by standard procedures (Harlow & Lane, 1988). A control serum (T858) was raised against a GST fusion protein which contained

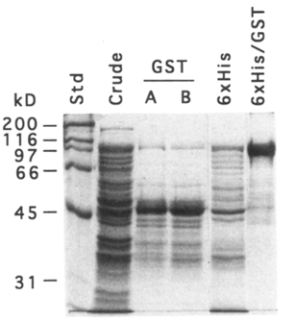

FIGURE 2: Purification of AK1-6H and Com-7. A Coomassie-stained SDS–10% polyacrylamide gel shows the improved purification obtained by using an affinity sandwich purification strategy. Arrow on the right side marks the predicted position of the AK1-6H fusion protein. Lanes are designated as follows: std, molecular weight standards; curde, 50 μg of *E. coli* cleared lysate containing AK1-6H before affinity chromatography; GST A, 10 μg of a Com-7 purification obtained from a single GST purification; GST B, 10 μg of an AK1-6H purification obtained from a single GST affinity step; 6 × His, 10 μg of an AK1-6H purification obtained from a single 6×His affinity step; 6×His/GST, 10 μg of purified AK1-6H obtained from a sequential 6×His and GST affinity purification.

sequences unrelated to the kinase. IgGs from both antisera were purified using protein G affinity chromatography with a MAb Trap GII column (Pharmacia, Alameda, CA) and concentrated. Antibodies were concentrated, and buffer was replaced with 50 mM Tris-HCl (pH 7.4) using a series of concentrations and dilutions in a Centricon 100 tube.

RESULTS

Affinity Sandwich Expression System. The first objective of this research was to develop a reliable expression system to provide highly purified AK1 fusions suitable for biochemical characterization of mutant activities. Preliminary investigations using construct Com-7 (Harper et al., 1993), which was a full-length AK1 fused to glutathione S-transferase (GST), indicated that a single affinity purification step was not sufficient to yield pure protein (see Figure 2). In some preparations, truncated proteins with kinase activity appeared to be present (data not shown). To overcome this problem, we added a six-histidine motif (6×His) to the C-terminal end of this AK1 fusion. This new affinity sandwich fusion was called AK1-6H (see Figure 1). Only partial purification was observed with either a single GST or 6×His purification step. However, a dramatic improvement was achieved when both 6×His and GST affinity selections were employed in series. This purification strategy gave AK1-6H enzyme, which was estimated to be greater than 95% pure (B. Binder, personal communication). The relative absence of truncation products was shown by Western blot analysis (Figure 4) using a monoclonal antibody which recognizes AK1.

AK1-6H Kinase Activity. The *E. coli* expressed AK1 displayed kinase activity similar to that of a native CDPK purified to homogeneity from soybeans (Harmon et al., 1994). The kinase activity of AK1-6H was stimulated by Ca^{2+} (up to 127-fold) with a typical preparation of enzyme displaying a specific activity of 2000 nmol min^{-1} mg^{-1}, using 100 μM syntide-2 and 300 μM ATP as substrates (Table 1). The native soybean enzyme displayed similar calcium stimulation but had a slightly higher specific activity of 9300 nmol min^{-1} mg^{-1}. Although this higher activity may be an isoform-specific difference, one AK1-6H preparation gave a specific activity of 4250 nmol min^{-1} mg^{-1}, which indicates that our typical *E. coli* expressed AK1 is not fully active. This single high specific activity AK1 was expressed and purified from *E. coli* which were grown very slowly under high osmotic stress (Blackwell & Horgan, 1991). Nevertheless, the range of specific activities observed between independent preparations of the same enzyme (including those for mutants) was always less than or equal to 2.3-fold when the enzyme was expressed and purified under standard conditions. Table 1 presents the activities of typical preparations.

Mutational Analysis of AK1-6H. The second objective of this research was to test the hypothesis that CDPKs are regulated by an autoinhibitory domain which is located outside the kinase catalytic core. The approach taken was to first generate mutants with deletions of the N- and C-terminal regions and test them for Ca^{2+}-independent kinase activity. Site-specific mutations in the junction domain were then used to confirm the location of the autoinhibitor in the full-length enzyme. All mutants were purified to near homogeneity by the affinity sandwich strategy except ΔJC, which required a different procedure. Deletion mutants are diagrammed in Figure 3, and their relative purity by Coomassie stain and Western blot analysis is documented in Figure 4.

Deletion of the N-Terminal Domain. Ca^{2+}-regulated activity did not appear to be altered in mutant ΔN-6H, which has a deletion of the first 124 amino acids (Table 1). Ca^{2+} stimulated ΔN-6H specific activity 41-fold to around 2000 nmol min^{-1} mg^{-1}, which was equivalent to that of AK1-6H.

Deletion of the Calmodulin-like Domain. Deletion of the calmodulin-like domain resulted in an enzyme which was low in activity (i.e., less than 2% of the fully stimulated AK1-6H; see Table 1). ΔC-6H enzyme was not stimulated by Ca^{2+}, and its basal activity was very low, with specific activities of multiple preparations ranging from 19 to 45 nmol min^{-1} mg^{-1}.

The low activity of ΔC-6H appears to be due to autoinhibition, since it can be activated by using antibodies raised against the junction domain (Figure 5). ΔC-6H enzyme was preincubated with purified IgG from antiserum T857 (anti-junction) or T858 (control). The T857 IgG at 666 μg mL^{-1} (40 μg per reaction) activated the ΔC-6H 87-fold to a specific activity of 3790 nmol min^{-1} mg^{-1}. A control IgG (T858), purified from antiserum raised against a nonkinase GST fusion, did not activate the enzyme when used at the same concentration. The anti-junction IgG (T857) also fully activates AK1-6H in the absence of Ca^{2+} but has no effect on the constitutively active ΔJC enzyme, which lacks a junction domain (data not shown). One hypothesis to explain the relatively high specific activity of the IgG-activated ΔC-6H (compared to AK1-6H at 2000 nmol min^{-1} mg^{-1}) is that ΔC-6H may retain its full latent activity because it can be expressed as a more stable enzyme in *E. coli*. This speculation is based on the idea that ΔC-6H's low basal activity is tolerated by *E. coli* and does not induce a toxic response as does the expression of AK1-6H (data not shown).

Deletion of the Junction and Calmodulin-like Domains. In contrast to the autoinhibited activity of ΔC-6H, a deletion of both the junction and the calmodulin-like domains resulted in a constitutively active enzyme (Table 1). Ca^{2+}-independent activity was observed for construct ΔJC, which has a deletion of all residues distal to amino acid D_{411}.

To purify a ΔJC to the same degree as an affinity sandwich purified AK1-6H, we employed Q-Sepharose chromatography following GST affinity purification. Figure 4 shows ΔJC eluted in three salt fractions. The Western blot shows that the purified ΔJC is not contaminated by truncation products, which would lower the molecular mass by more than 10 kD.

Table 1: Specific Activities for Purified AK1 Fusion Proteins Expressed in *E. coli*

	specific activity[a] (nmol min^{-1} mg^{-1})		X-fold calcium activation	activity[b] vs AK1-6H (%)	enzyme description[c]
	$-Ca^{2+}$	$+Ca^{2+}$			
AK1-6H (av)[d]	30	2000	67	100	wild-type model
AK1-6H (high)	42	4250	101	212	"BS media" prep[f]
truncations					
ΔN-6H	50	2070	41	100	Δaa M_1-H_{124}
ΔC-6H	19	21	1.1	1	trunc at A_{454} + 6H
ΔJC (0.3 M)[e]	1780	1490	0.8	89	trunc at D_{411}
ΔJC(26)-6H	1140	950	0.8	57	ΔJC + 53 aa + 6H
J mutations					
KJM23-6H2	1720	1940	1.1	86	mutation 6 aa in J
KJM25-6H2	215	266	1.2	10	mutation 11 aa in J
KJM26-6H4	435	227	0.5	22	J sub with 30 aa
KJM26-6H6	429	309	0.9	21	J sub with 51 aa
KJM13-6H	16	2060	128	100	9 aa between K and J

[a] Specific activities reported are from a typical enzyme preparation and represent the average from at least four assays. Our assay system displayed an average variation of ±6% for activity measurements of any given enzyme preparation. However, activity measurements varied up to 2.3-fold between preparations. At least two enzyme preparations were tested for each construct, except ΔN-6H and KJM13-6H, which were purified only once. Because of the low activity of ΔC mutants, more than 10 enzyme preparations were tested, and all gave equivalent results. [b] AK1-6H (av) was used as reference. For comparison to Ca^{2+}-independent mutants, activities used for mutants were those measured under minus Ca^{2+} conditions. [c] Abbreviations used: Δ, deletion including residues; aa, amino acid; trunc, truncation point after indicated residue; +6H, addition of 6xHis motif; sub, substitution; K, kinase domain; J, junction domain; C, calmodulin-like domain. [d] AK1-6H (av) was calculated as an average of six typical purifications. The highest Ca^{2+} stimulation was 127×. [e] ΔJC (0.3 M) was purified by Q-Sepharose chromatography. The purified fraction assayed was eluted with 0.3 M NaCl. [f] *E. coli* were grown in "BS media", which is 2×YT supplemented with 1 M sorbitol and 2.5 mM betaine.

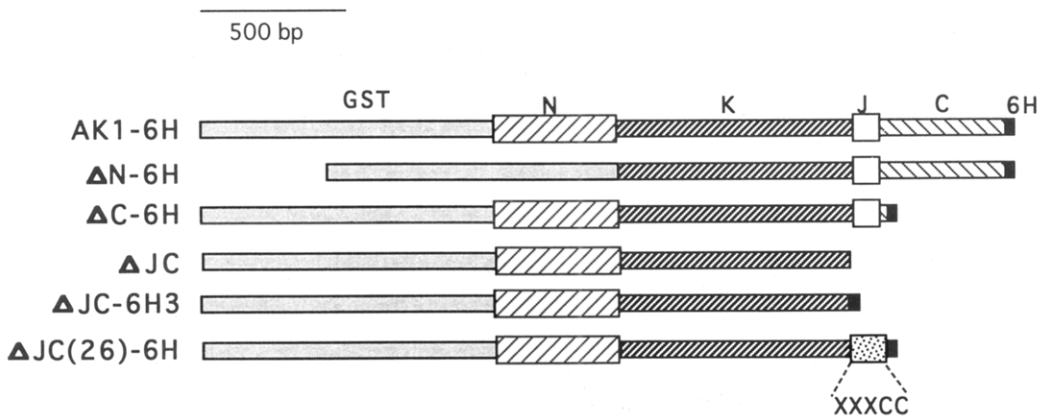

FIGURE 3: Diagram of truncation mutants. The structure of N- and C-terminal truncation mutants are shown relative to AK1-6H. The XXXCC insert in ΔJC(26)-6H represents three tandem Factor Xa sites and two tandem c-myc epitopes. The 6×His motifs at the end of the genes are marked as a black box. ΔJC does not have a terminal 6×His motif.

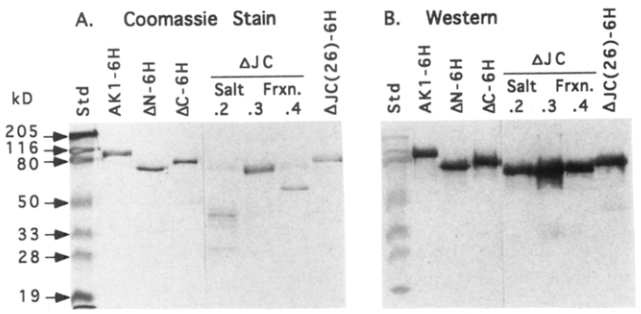

FIGURE 4: Purification of truncation mutants. (A) a Coomassie-stained SDS–10% polyacrylamide gel loaded with 2 μg per lane of purified enzyme from each truncation mutant. For ΔJC, three salt fractions obtained after Q-Sepharose ion-exchange chromatography are shown. All other proteins were purified by an affinity sandwich strategy. (B) Western blot of an identical gel loaded with 0.2 μg of each protein. Proteins were immunodecorated with a mouse anti-CDPK monoclonal antiserum which was detected by an alkaline phosphatase-coupled anti-mouse secondary antibody. Standards shown were prestained markers from Bio-Rad.

The purity of ΔJC (0.3 M fraction) was comparable to that of AK1-6H, as confirmed by immunological quantitation of a parallel dilution series of both enzymes, using a CDPK monoclonal antibody for detection (data not shown).

The specific activity of ΔJC (0.3 M salt fraction) was approximately 89% that of a fully stimulated AK1-6H. This value is closer to 70% when the specific activity is corrected for differences in molecular mass and reported on a per mole basis (ΔJC is approximately 20% smaller than AK1-6H).

The activity of ΔJC was confirmed by a related mutant ΔJC(26)-6H. A potential problem of ΔJC not having a C-terminal 6×His tag was that its predicted C-terminus was not easily verified. This was considered important since small proteolytic clips at this position could significantly alter the activity of the enzyme. Therefore, we engineered a related enzyme, ΔJC(26)-6H, which is truncated at the same point as in ΔJC but has a 6×His tag included at the end of a 61-residue extension. Both enzymes were Ca^{2+}-independent, although ΔJC(26)-6H preparations were consistently less active, with only 57% the activity of AK1-6H (Table 1).

To generate a functional 6×His affinity tag at the end of a ΔJC construct, it was found that the tag must be placed at the end of a long extension (e.g., 61 amino acids in ΔJC-(26)-6H). 6×His affinity chromatography failed to purify

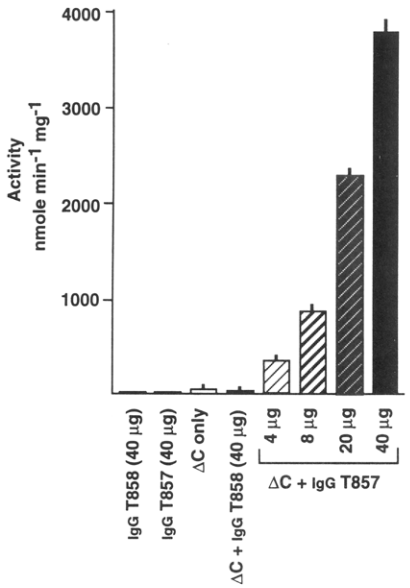

FIGURE 5: ΔC-6H activation by anti-junction antibodies. The specific activity of ΔC-6H is shown when assayed with and without an anti-junction antibody. Standard 60-μL kinase reactions with 0.05 μg of ΔC-6H enzyme were preincubated with 0–40 μg of purified IgG for 1 h at 22 °C and initiated by the addition of 300 μM ATP. IgG T858 was purified from a control antisera raised against a "non-kinase" GST fusion protein. IgG T857 was purified from antisera raised against a GST/junction domain fusion. The ΔC-6H preparation used here had a basal specific activity of 43.8 ± 1.3 (n = 2) nmol min^{-1} mg^{-1}. Activities were calculated from the average of duplicate assays. A second ΔC-6H preparation was assayed with equivalent results.

three ΔJC-related truncation mutants: ΔJC-6H1, ΔJC-6H3, and ΔJC-6H4 (data not shown). The 6×His tags in these constructs were all within 21 residues of D_{411}, immediately following the kinase domain. An explanation for this failure is that sequences in this region are constrained or tightly associated with the kinase domain. This hypothesis is supported by a second example. In construct KJM13-6H, a Factor Xa protease cleavage site was engineered at the end of the kinase domain. This site failed to be efficiently cleaved by Factor Xa protease. However, the site was cleaved when its position was shifted to the C-terminal side by the insertion of 18 additional residues (data not shown). Therefore, two empirical observations indicate that peptide sequences located at the end of the kinase domain (and at the beginning of the junction domain) may be inaccessible to large macromolecules.

The kinetic parameters of ΔJC were examined to determine if its Ca^{2+}-independent activity was due to a disruption of autoinhibition or a mutation of the catalytic mechanism which altered the K_m values for substrates. The apparent K_m values for ATP and syntide-2 were measured in the presence and absence of Ca^{2+} (Table 2). Under both conditions, the K_m values were equivalent to those measured for a Ca^{2+}-stimulated AK1-6H. Thus, the only significant difference between AK1-6H and ΔJC is that ΔJC activity does not require Ca^{2+}. In fact, Ca^{2+} actually causes a 10–50% inhibition of ΔJC activity. A similar Ca^{2+} inhibition was observed for truncation mutants of MLCK (Ito et al., 1991).

Junction Domain Mutations. To confirm that the junction domain functioned as an autoinhibitor in a full-length enzyme, this region was subjected to site-specific mutagenesis. The four mutants described here are KJM23-6H, KJM25-6H, KJM26-6H4, and KJM26-6H6 (see Figure 6 for diagrams, Figure 7 for documentation of relative purity, and Table 1 for activity information). All four had mutations which disrupted the autoinhibitor and resulted in Ca^{2+}-independent enzymes.

Table 2: Apparent K_m Values for ATP and Syntide-2 Substrates for AK1-6H Compared to Ca^{2+}-Independent Mutants ΔJC and KJM23-6H

	K_m ATP[a] (μM)		K_m syntide-2[b] (μM)		specific activity (nmol min^{-1} mg^{-1})	
enzyme	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$
AK1-6H	nd[c]	44 ± 4	nd[c]	49 ± 5	20	2800
ΔJC	45 ± 6	45 ± 4	55 ± 4	54 ± 5	1780	1490
KJM23-6H	50 ± 2	38 ± 2	92 ± 16	63 ± 9	1750	1940

[a] Syntide-2 was held constant at 100 μM, and activity was measured in duplicate assays with ATP concentrations of 0, 20, 50, 100, 150, 200, and 500 μM under standard conditions. K_m values were calculated from Lineweaver–Burke plots. Computer fit and hand-drawn plots gave equivalent results. [b] ATP was held constant at 300 μM, and activity was measured in duplicate assays with syntide-2 concentrations of 0, 20, 40, 60, 80, 100, and 125 μM under standard conditions. K_m values were calculated from Lineweaver–Burke plots as described above. [c] Not determined. Activity of AK1-6H was near background in the absence of Ca^{2+}.

The most active Ca^{2+}-independent mutant obtained was KJM23-6H, which had a substitution of six amino acids in the central region of the junction domain (i.e., between A_{422} and A_{432}). The activity of this enzyme was 86% that of AK1-6H. A small but reproducible 1.1-fold activation by Ca^{2+} was observed. The K_m for ATP ranged from 38 to 50 μM for plus and minus Ca^{2+} conditions, respectively, and the K_m values for syntide were 60 μM in the presence of Ca^{2+} and 93 μM in the absence of Ca^{2+} (Table 2). These values are very close to the values obtained for ΔJC and a fully activated AK1-6H. It is possible that the slightly higher K_m for syntide and ATP observed under minus Ca^{2+} conditions explains the marginal Ca^{2+} stimulation that was seen with this enzyme.

Mutants KJM25-6H, KJM26-6H4, and KJM26-6H6 each had at least 11 amino acids replaced by a c-myc epitope. The c-myc epitope was used as a nonspecific replacement sequence. In comparison to KJM23-6H, each represents a progressively more drastic change to the junction domain. While all of these mutants were Ca^{2+}-independent, none was as active as KJM23-6H. This may indicate that the c-myc epitope sequence when located in the central region of the junction domain provided partial autoinhibition and was not totally inert. This hypothesis would also explain the lower activity of ΔJC(26)-6H (which contains a c-myc epitope in the same position) compared to ΔJC. The c-myc epitope does contain a KLIS sequence, which may be recognized as a kinase substrate or a pseudosubstrate, since it conforms to a consensus substrate site of basic-X-X-S/T (Roberts & Harmon, 1992). An alternative hypothesis is that the lower specific activities are due to a destabilization of the enzymes as a result of the c-myc epitope's structures or location.

In contrast to mutations disrupting the central region of the junction domain, one mutation, called KJM13-6H, was made which had an insertion of nine amino acids at the beginning of the junction domain. KJM13-6H displayed activity similar to that of AK1-6H in both Ca^{2+} stimulation and specific activity (Table 1). This mutant indicates that an exact alignment of the autoinhibitor in the junction domain does not appear to be critical for its function.

Peptide Inhibitors. The third objective of this investigation was to explore the mechanism by which the autoinhibitor functions. Since the mutational studies supported a structural analogy between CDPK and Ca^{2+}/calmodulin-dependent protein kinases, we decided to test whether AK1-6H and ΔJC activity could be inhibited by known peptide inhibitors of

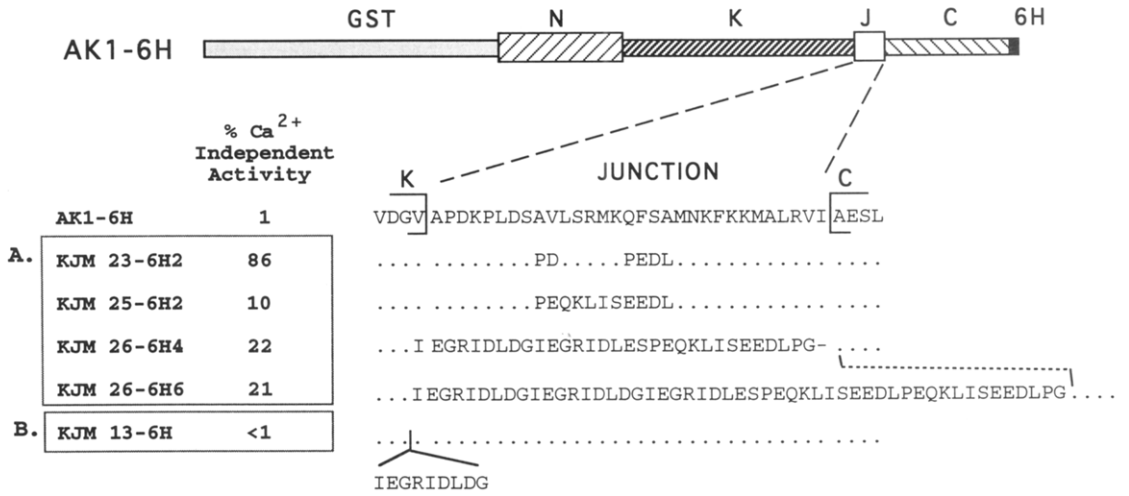

FIGURE 6: Diagram of junction mutations. The sequence of the junction domain and engineered mutations are shown under a diagram of AK1-6H. Unchanged residues are denoted by a dot. A dash indicates the absence of a residue. All mutants identified in box A have mutations which disrupt the central region of the junction domain and generate Ca^{2+}-independent enzymes. The mutant in box B has an insertion immediately preceding the junction domain. The percent Ca^{2+}-independent activity is relative to a specific activity of 2000 nmol min^{-1} mg^{-1} obtained for a typical AK1-6H preparation after Ca^{2+} stimulation.

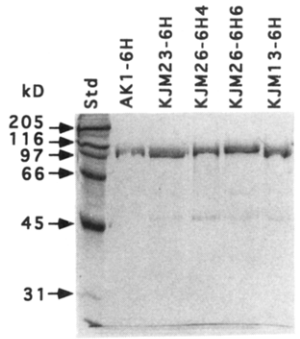

FIGURE 7: Purification of junction mutants. A Coomassie-stained SDS–10% polyacrylamide gel is shown loaded with 2 μg per lane of purified enzyme from each junction mutant. Purified protein for KJM25-6H was not included on this gel but was of equivalent purity when evaluated in a separate gel.

skMLCK (Kawasaki et al., 1993) and CaMKIIβ (Payne et al., 1988) (Figure 8). The sequences of the two peptides examined were based on their respective pseudosubstrate-type autoinhibitors. Both peptides inhibited AK1-6H and ΔJC with IC_{50} concentrations which ranged between 3 and 30 μM under our standard assay conditions (Figure 9 shows inhibition of ΔJC).

ΔJC and AK1-6H were also inhibited by a third peptide, melittin, at IC_{50} concentrations between 20 and 25 μM, but only when lipids were not included in the standard assay reaction. Although melittin was originally chosen for study because of its ability to bind calmodulin in a Ca^{2+}-dependent fashion (Kincaid & Coulson, 1985; Baudier et al., 1987), it also has a high affinity for certain lipids such as phosphatidylserine, and slightly less for phosphatidylcholines (Faucon et al., 1979; Dufourcq & Faucon, 1977; Mollay & Kreil, 1973).

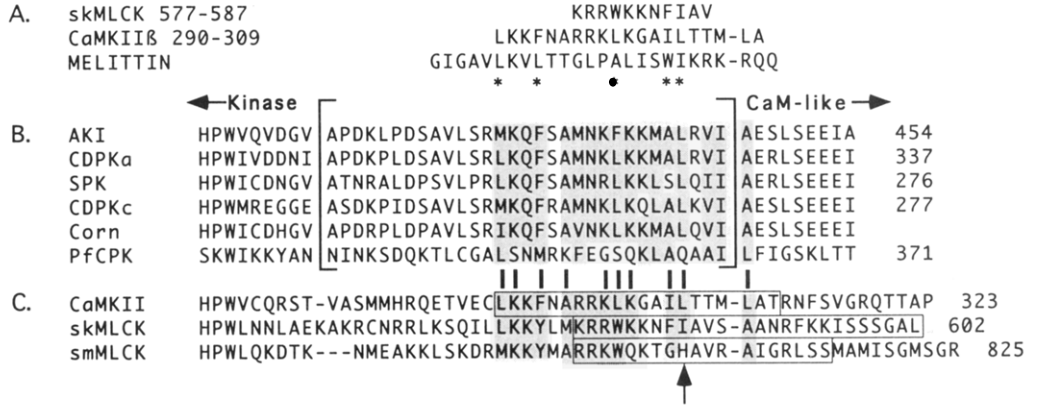

FIGURE 8: Alignment of peptide inhibitors, CDPK junction domains, and autoinhibitory domains of CaMKIIβ, skMLCK, and smMLCK. (A) Peptides used for inhibition of ΔJC activity are aligned above CDPK junction domains. skMLCK 577–587 (Kawasaki et al., 1993) and CaMKIIβ (Payne et al., 1988) are known inhibitors of their respective kinases. Melittin is a lipid binding peptide which also binds to calmodulin in a Ca^{2+}-dependent fashion (O'Neil & DeGrado, 1990). Sites of hydrophobic residues which are shared between peptides and ΔJC are marked by an asterisk. (B) Alignment of junction domains from plant and protist CDPKs. Shaded residues represent positions of similar amino acids in a comparison of CDPKs. Kinase sequences (in B and C) are aligned starting with a conserved motif "HPW" located near the end of the kinase domain. CDPK isoforms presented are AK1 (*Arabidopsis*; Harper et al., 1993), CDPKα (soybean; Harper et al., 1991), SPK (rice, Kawasaki et al., 1993), CDPKc (carrot; Suen & Choi, 1991), corn (partial corn cDNA; Poovaiah & Reddy, 1993), and PfCPK (*Plasmodium*, Zhao et al., 1993). The junction domain is bracketed apart from the kinase domain and the calmodulin-like (CaM-like) domain. Numbers on the right refer to the position of the last residue of the protein sequence shown. (C) Alignment of autoinhibitory domains of Ca^{2+}/calmodulin-dependent protein kinases from skMLCK (Takio et al., 1986), smMLCK (Shoemaker et al., 1990), and CaMKIIβ (Bennet & Kennedy, 1987). Shaded residues represent positions of similar amino acids in a comparison of CaMKIIβ, skMLCK, and smMLCK. Boxed sequences correspond to peptides which have been shown to bind to calmodulin in a Ca^{2+}-dependent fashion (O'Neil & DeGrado, 1990). An arrow indicates the H residue in smMLCK thought to be the residue in the pseudophosphorylation site (Knighton et al., 1992). Similar residues between CDPKs (B) and Ca^{2+}/calmodulin-dependent protein kinases (C) are shown by bold lines between the two groups.

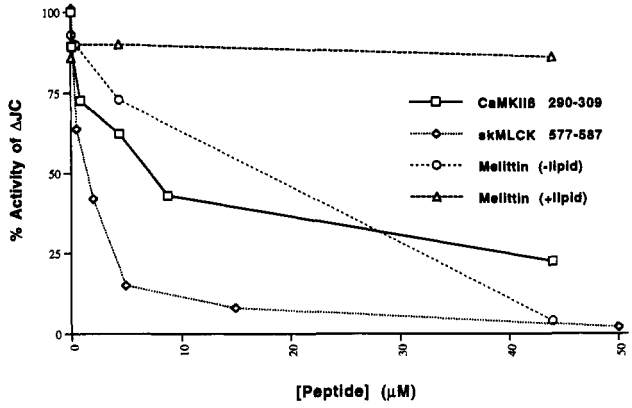

FIGURE 9: Inhibition of ΔJC by pseudosubstrate and calmodulin binding peptides. A dose response is shown for inhibition of ΔJC by peptides skMLCK 577–587, CaMKIIβ 290–309, and melittin. The sequences of these peptides are shown in Figure 8. The ΔJC enzyme used here had a specific activity of 1700 nmol min^{-1} mg^{-1}. Peptides were tested under standard assay conditions with and without lipids. Lipids had no effect on the inhibition by skMLCK 577–587 and CaMKIIβ 290–309. However, 1 mg mL^{-1} crude lipid reversed the inhibitory properties of melittin. Each point represents the average of at least two assays. These studies were repeated with the full-length enzyme AK1-6H with similar results (not shown).

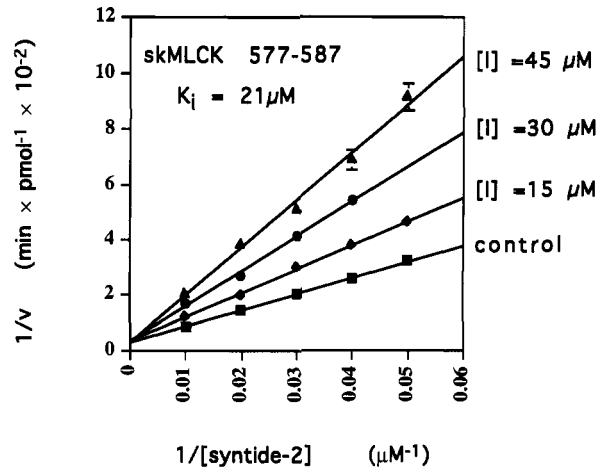

FIGURE 10: Double reciprocal plot of ΔJC inhibition by skMLCK 577–587 peptide. The kinase activity of ΔJC was evaluated under standard assay conditions without Ca^{2+} or lipid. Syntide-2 concentrations used were 20, 25, 33, 50, and 100 μM in the presence of peptide inhibitor at concentrations of 0, 15, 30, and 45 μM. The plot was made with the CA-Cricket Graph III computer software, and lines were generated on the basis of the data using the linear regression fit function provided by the program. A K_i of 21 ± 2 μM was calculated from the slopes of the four fitted lines.

The inhibitory properties of melittin were reversed by the addition of crude phosphatidylcholine in our assays. It is likely that melittin formed a peptide/lipid complex which reduced the peptide's effective concentration. Lipid did not appear to affect the inhibitory properties of the MLCK and CaMKII peptides (data not shown).

The observed peptide inhibition of ΔJC suggests that the three peptides examined may have a direct interaction with the kinase domain. However, in the case of AK1-6H activity, inhibition could also involve an interaction of the peptides with the calmodulin-like domain, since all three peptides have been shown to bind calmodulin in a Ca^{2+}-dependent fashion (O'Neil & DeGrado, 1990). This alternative mode of action is not relevant to ΔJC since it has a truncation of the calmodulin-like domain.

The inhibition by the skMLCK peptide was chosen for further characterization because this peptide appeared to be the most potent inhibitor, based on its lower IC_{50} (3 μM with ΔJC). Standard kinase assays were performed with ΔJC using varying concentrations of syntide-2 and peptide skMLCK 577–587. Inhibitory kinetics were analyzed by double reciprocal plots. As shown in Figure 10, the peptide inhibited ΔJC in a competitive fashion, with respect to syntide-2, with a K_i of 21 ± 2 μM.

DISCUSSION

The fusion protein AK1-6H appears to represent a valid model for investigating the mechanisms by which Ca^{2+} regulates CDPK activity. The kinase activity of purified AK1-6H was stimulated as much as 127-fold by Ca^{2+} under standard assay conditions with syntide-2 as substrate. This level of activation is equivalent to that of a native CDPK enzyme purified from soybean (Harmon et al., 1987; Putnam-Evans et al., 1990). Therefore, the Ca^{2+} regulatory mechanism in AK1-6H is retained in this fusion protein, despite modification of its N- and C-terminal ends.

Affinity Sandwich Purification System. To conduct an extensive mutational analysis of AK1, it was necessary to develop a method to rapidly purify mutant enzymes to near homogeneity. Previous research indicated that AK1 did not express well in *E. coli*, and purification of full-length protein by a single affinity chromatography step was not possible. Here, a dramatic improvement in purification was achieved by constructing a fusion which sandwiched the AK1 kinase between a glutathione S-transferase and six consecutive histidines at the N- and C-terminal ends, respectively. This arrangement permitted purification based on two sequential affinity chromatography steps, a strategy we termed affinity sandwich purification. An important advantage of this strategy is that it selects for full-length fusions and removes proteins with truncated N- or C-terminal ends. This was important since certain truncations of the C-terminal end (i.e., ΔJC) significantly altered the AK1 kinase activity. In comparison to earlier studies (Harper et al., 1991), AK1-6H displayed a 50-fold higher specific activity of around 2000 nmol min^{-1} mg^{-1}. This increase is primarily due to improvements in purification, with additional increases resulting from optimized assay conditions and the use of syntide-2 as a substrate (rather than histone type IIIs).

Unique N-Terminal Domain. The function of a 15-kD N-terminal domain in AK1 is not known but is of interest because it is not present in other published CDPKs. The level of Ca^{2+} stimulation observed for AK1-6H was not affected in a mutant ΔN-6H which has a deletion of its unique N-terminal domain (i.e., the first 124 amino acids). This indicates that it is not critical to Ca^{2+} activation *in vitro*, which confirms a previous report based on a partially purified N-terminal truncation, KGF-1 (Harper et al., 1991). Our current speculation is that the N-terminal domain may be important for intracellular localization or substrate specificity.

Location of Autoinhibitory Sequence. Truncation of AK1-6H at amino acid D_{411} deletes the junction and calmodulin-like domains and generates an active Ca^{2+}-independent enzyme. This indicates that an autoinhibitory function is located distal to the end of the kinase domain and verifies that the autoinhibitor is not a property of the kinase catalytic core. Therefore, CDPKs do not appear to be regulated by an allosteric mechanism analogous to that proposed for CDC2, where autoinhibition is an intrinsic feature of the catalytic core. CDPKs also appear to be different from protein kinase C, which has an autoinhibitor located to the N-terminal side of the kinase domain (House & Kemp, 1987). Therefore, the

most appropriate paradigm for CDPK appears to be Ca^{2+}/calmodulin-dependent protein kinases such as CaMKII and MLCK. Both of these enzymes have pseudosubstrate-type autoinhibitors located to the C-terminal side of the kinase domain and can be converted into unregulated kinases by truncations which are analogous to a ΔJC truncation described here for AK1 (Hagiwara et al., 1991; Pears et al., 1990; Planas-Silva & Means, 1992; Ito et al., 1991; Shoemaker et al., 1990).

The contention that CDPKs have an autoinhibitor located in the junction domain rather than the calmodulin-like domain is supported by two lines of genetic evidence. First, ΔC mutants, which lack a calmodulin-like domain but retain a junction domain, are inactive. The low activity of a ΔC mutant is consistent with the junction domain keeping the enzyme inhibited. The observation that antibodies against the junction domain can fully activate ΔC-6H verifies that this mutant is kept inactive due to autoinhibition rather than being "dead" due to misfolding or instability. An artificial activatation of protein kinase C has also been reported which was accomplished by using antibodies raised against its pseudosubstrate autoinhibitor (Makowske & Rosen, 1989). Second, four site-specific mutants, which had the central portion of the junction domain disrupted, all produced Ca^{2+}-independent enzymes with activities approximately 10–86% that of AK1-6H. The most active mutant was KJM23-6H, which had a substitution of six amino acids between residues A_{422} and A_{432}. The lower activity of other junction mutations may indicate that partial autoinhibition can be obtained from nonhomologous sequences (in this case, a c-myc epitope) located in the position of the junction domain.

Evidence for Pseudosubstrate-type Autoinhibitor. Our mutational analysis indicates that the junction domain contains an autoinhibitor. The autoinhibitor was proposed to function as a pseudosubstrate since its location is analogous to the position of pseudosubstrate-type autoinhibitors in Ca^{2+}/calmodulin-dependent protein kinases (e.g., MLCK and CaMKII) (Harper et al., 1991, 1993; Roberts & Harmon, 1992). This prediction was corroborated by peptide inhibitor studies in the laboratory of Alice Harmon (Harmon et al., 1994). A peptide based on a junction domain sequence (residues 310–332 of CDPKα) was shown to competitively inhibit, with respect to syntide-2, the activity of a native CDPK purified from soybean (K_i = 5 μM). This peptide also competitively inhibited a ΔJC-like enzyme [engineered from a soybean cDNA (CDPKα)] with a similar K_i of around 5 μM.

Here we extend the peptide inhibitor studies to show that peptide sequences derived from the pseudosubstrate autoinhibitory domains of skMLCK and CaMKIIβ can also function as inhibitors of CDPK isoform AK1. Their IC_{50}s for inhibition of AK1-6H and ΔJC ranged from 3 to 30 μM. The skMLCK peptide was examined in greater detail and was shown to inhibit ΔJC with a K_i of 21 μM in a competitive fashion with respect to syntide-2. This is consistent with the idea that CDPK evolved from a Ca^{2+}/calmodulin-dependent protein kinase (Harper et al., 1991) and has retained a similar mechanism of autoinhibition.

It is not clear which residues and structural features are important for the autoinhibitor's function. The observation that our ΔJC is inhibited by the peptide melittin raises the possibility that a general structure, such as a basic amphipathic α-helix, rather than a specific peptide sequence may be the important feature of the autoinhibitory domain. This hypothesis is supported by studies on phosphorylase kinase which also show that melittin can inhibit this Ca^{2+}/calmodulin-dependent enzyme by an interation with its catalytic center (Paudel et al., 1993). A common property of all of the peptide inhibitors discussed here is that they can bind calmodulin in a Ca^{2+}-dependent fashion and form amphipathic α-helices. Although they also contain multiple basic residues, the importance of basic residues for the autoinhibitor function has been questioned for MLCK (Herring, 1991; Yano et al., 1993).

Regulatory Models. This investigation on isoform AK1, together with the peptide inhibitor studies by A. Harmon on soybean CDPKs, provides strong experimental support for the model that a pseudosubstrate-type autoinhibitor is located in the junction domain. With respect to enzyme regulation, the next important challenge is to understand how the autoinhibitor is displaced when the calmodulin-like domain binds Ca^{2+}. Two general mechanisms of stimulation are consistent with the unique structural arrangement of CDPKs. First, by analogy to calmodulin-dependent enzymes, Ca^{2+} may induce an intramolecular "binding" of the calmodulin-like domain to the junction domain, thus interfering with the ability of the junction domain to bind to the catalytic site and inhibit kinase activity. Second, in an alternative mechanism, a conformational change in the calmodulin-like domain could directly "pull" or "twist" the junction out of its site of autoinhibition without a specific intramolecular "binding" reaction. This alternative appears reasonable since calmodulin-like proteins undergo significant Ca^{2+}-induced conformational changes and, in the case of CDPK, the calmodulin-like domain is already directly coupled to the autoinhibitory sequence.

Since each of these potential mechanisms represents a novel departure from known Ca^{2+}-regulated protein kinases, an intriguing question is whether this family of kinases is uniquely suited for special physiological purposes. For example, in comparison to a Ca^{2+}/calmodulin-dependent protein kinase, can CDPKs respond to a more rapid Ca^{2+} pulse? This may be possible since Ca^{2+} activation of CDPK would not be limited by a relatively slow diffusion of a Ca^{2+}/calmodulin complex. With the recent demonstration that Ca^{2+}/calmodulin-dependent protein kinases exist in plants (Watillon et al., 1993), the potential exists that plants utilize these two distinct kinase pathways to interpret different Ca^{2+} signals.

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