Evidence for Differing Roles for Each Lobe of the Calmodulin-like Domain in a Calcium-dependent Protein Kinase*§

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Calcium-dependent protein kinases (CDPKs) are structurally unique Ser/Thr kinases found in plants and certain protozoa. They are distinguished by a calmodulin-like regulatory apparatus (calmodulin-like domain (CaM-LD)) that is joined via a junction (J) region to the C-terminal end of the kinase catalytic domain. Like CaM, the CaM-LD is composed of two globular EF structural domains (N-lobe, C-lobe), each containing a pair of Ca$^{2+}$ binding sites. Spectroscopic analysis shows that the CaM-LD is comprised of helical elements, but the isolated CaM-LD does not form a conformationally homogeneous tertiary structure in the absence of Ca$^{2+}$. The addition of substoichiometric amounts of Ca$^{2+}$ is sufficient to stabilize the C-terminal lobe in a construct containing J and CaM-LD (JC) but not in the CaM-LD alone. Moreover, as J is titrated into Ca$^{2+}$-saturated CaM-LD, interactions are stronger with the C-lobe than the N-lobe of the CaM-LD. Measurements of Ca$^{2+}$ affinity for JC reveal two cooperatively interacting high affinity binding sites ($K_{d,mean} = 5.6$ nM at 20 mM KC1) in the C-lobe and two weaker sites in the N-lobe ($K_{d,mean} = 110$ nM at 20 mM KC1). The corresponding Ca$^{2+}$ binding constants in the isolated CaM-LD are lower by more than 2 orders of magnitude, which indicates that the J region has an essential role in stabilizing the structure of the CDPK regulatory apparatus. The large differential affinity between the two domains together with previous studies on a plasmodium CDPK (Zhao, Y., Pokutta, S., Maurer, P., Lindt, M., Franklin, R. M., and Kappes, B. (1994) Biochemistry 33, 3714-3721) suggests a model whereby even at normally low cytosolic levels of Ca$^{2+}$, the C-lobe interacts with the junction, but the kinase remains in an autoinhibited state. Activation then occurs when Ca$^{2+}$ levels rise to fill the two weaker affinity binding sites in the N-lobe, thereby triggering a conformational change that leads to release of the autoinhibitory region.

Calcium-dependent protein kinases (CDPKs) participate in signal transduction events at the intersection of Ca$^{2+}$ and phosphorylation-dependent signaling pathways. CDPKs are found only in plants and protozoa, including Plasmodium falciparum, the causative agent of malaria (1-5). Biochemical features of CDPK substrates suggest regulatory roles for CDPKs in gene expression, metabolism, signaling pathway components, ion transport, and cytoskeleton dynamics (1, 2, 5).

On the basis of sequence homology, CDPKs are most closely related to calmodulin-dependent protein kinases (CaMKs) (6, 1). The majority of CaM-regulated kinases contain a CaM binding region near their C terminus. The CDPKs are similar, except they contain their own calmodulin-like regulatory apparatus (CaM-LD) C-terminal to the CaM binding region of the catalytic domain (K) (Fig. 1). The CaM-LD consists of two structural domains (termed the N and C “lobes” in this manuscript), each containing two EF-hand helix-loop-helix Ca$^{2+}$ binding motifs. The region that joins the kinase and CaM-LD is referred to as the junction (J). The junction contains an autoinhibitor and a target sequence for intramolecular binding of the CaM-LD (7).

The similarities between the CaMK and CDPK systems are numerous. For example, both CaMKs and CDPKs are regulated by an autoinhibitor located immediately C-terminal to the kinase domain. In both cases, autoinhibition is relieved by interaction with CaM or the CaM-LD. The CaM-LD of CDPK has 40% sequence identity with CaM, which suggests a potential similarity in structure and function. In fact, the CaM-LD can be functionally replaced by a CaM sequence in a chimeric CDPK (8). Additionally, both an exogenous CaM and a CaM-LD were shown to be able to activate a truncated CDPK, although to no more than half of the extent of the holoenzyme (9). However, what distinguishes CDPKs and CaMKs is the cova-
lent tethering of the CaM-LD in a CDPK. Evidence from muta-
tional analyses indicates that this tether provides a struc-
tural constraint that is essential for the mechanism of
intramolecular activation. An insertion of three glycines into
the tether, which would be expected to increase its flexibility,
results in a 95% disruption in calcium activation (9). The exact
structural features of the tether that help mediate activation
by the CaM-LD are unclear.

To more fully elucidate the mode of action of the regulatory
apparatus of CDPK, we have undertaken a series of biophysical
analyses of the CaM-LD and its interactions with the J region.
The structural character of the CaM-LD was examined by CD
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finding is consistent with previous studies on a plasmodium
CDPK isofrom (12) where mutational analysis showed that
only the N-lobe calcium sites were essential for calcium-de-
pendent activation.

EXPERIMENTAL PROCEDURES

Protein Expression—The CDPK isofrom, CPK-1, used in this work
was cloned from Arabidopsis thaliana (13). Constructs encoding the
regulatory apparatus of CDPK (residues Lys-428—Gly-591) were sub-
cloned from CPK-1 and sequenced to verify the absence of mutations
resulting from subcloning. The pET-28b/H11001 plasmid (Novagen) was used
for constructs of the CDPK regulatory domain. This has an N-terminal
His tag and a thrombin cleavage site and was used with Escherichia coli
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UV CD spectra in the absence and presence of Ca\(^{2+}\) for JC and the CaM-LD are shown in Fig. 2. As expected from the sequence and structural homology with CaM, the dominant contribution is from helical secondary structure (minima at 208 and 222 nm). These results are consistent with the analysis of the NMR chemical shifts of Ca\(^{2+}\)-loaded JC (16), which showed that the distribution of elements of secondary structure in CaM-LD (Fig. 1B) is very similar to that of CaM.

Although the CD spectrum of the Ca\(^{2+}\)-free state of JC clearly indicates considerable helical structure is present, its \(^{1}H,^{15}N\) HSQC spectrum is characterized by broad peaks and very limited chemical shift dispersion (Fig. 3). Together, the CD and NMR data indicate that the protein maintains much if not all of the native secondary structure but lacks a stable tertiary fold in the absence of Ca\(^{2+}\) (20). The addition of Mg\(^{2+}\) (20–100 mM) in an attempt to stabilize the tertiary structure of the apo protein had no effect on the NMR spectrum. These observations are consistent with the lack of an effect of Mg\(^{2+}\) on the binding of Ca\(^{2+}\) to soybean CDPK (8).

Significantly higher intensity is found in the CD spectrum of apoJC relative to that of apoCaM-LD (Fig. 2), implying that the J region in JC has partial helical character. Because the J peptide is unstructured on its own, this observation suggests that the CaM-LD interacts with the J region even in the absence of Ca\(^{2+}\). This contrasts with the tethered CaM-M13 (binding sequence from myosin light chain kinase) hybrid sys-

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**Fig. 1. Structural organization of CDPK regulatory apparatus.** A, schematic diagram of domain structure of CDPKs and constructs used in this study. N and C denote the N- and C-lobes of the CaM-LD, respectively. The black striped regions denote the Ca\(^{2+}\)-binding loops. B, amino acid sequence and secondary structure of the JC construct. The secondary structural elements indicated are derived from analysis of the NMR assignment of JC (16) using TALOS (18). β-Strand segments are underlined.

**Fig. 2. Far-UV CD spectra of the CDPK regulatory apparatus.** Spectra of JC and the CaM-LD in the absence and presence of Ca\(^{2+}\). Spectra were recorded with 5–10 μM protein in 2 mM Tris-HCl, 18 mM KCl, 0.1 mM DTT at pH 7.2.
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from surface plasmon resonance measurements (7). Spectra were recorded at 0.59 mM protein concentration in 50 mM Tris-d11 and 100 mM KCl with 10 mM DTT and 5% (v/v) D\(_2\)O at pH 7.2 and 310 K.

**Table I**

Macroscopic Ca\(^{2+}\) binding constants of CDPK JC and CaM-LD

| [KCl] \(\text{mM}\) | \(\log K_1\) | \(\log K_2\) | \(\log K_3\) | \(\log K_4\) |
|-----------------|------------|------------|------------|------------|
| JC\(^c\)        | 20         | 7.7 (+ 0.3 - 0.9) | 8.9 (+ 0.3 - 0.9) | 6.5 ± 0.1  | 7.4 ± 0.1  |
| CaM-LD\(^b\)    | 20         | 7.4 ± 0.1   | 5.7 ± 0.1   | < 4        | < 4        |
| CaM\(^b\)       | 25         | 5.8 ± 0.2   | 7.0 ± 0.2   | 5.4 ± 0.2  | 5.7 ± 0.2  |
| CaM\(^b\) hybrid\(^a\) | 100     | 4.9 ± 0.2   | 6.6 ± 0.2   | 4.4 ± 0.3  | 5.6 ± 0.3  |
| CaM/H13 hybrid\(^a\) | 100     | 8.0        | 7.4        | 7.8        | 7.8        |

\(^a\) \(K_1\) and \(K_2\) are shown to correspond to the C-lobe sites and \(K_3\) and \(K_4\) to N-lobe sites (see text for explanation). The domain-specific assignment is based on NMR analysis (see below). The same relative affinities are observed for the N- and C-lobes of CaM.

\(^b\) Taken from Linse et al. (27) at 298 K in 2 mM Tris-HCl, pH 7.5.

\(^c\) Taken from Martin et al. (21) at 293 K in 10 mM Tris, pH 8.0; WFF peptide (KKRWKKNFIAVSAANRFK, residues 1–18 of the M13 sequence).

**Fig. 3.** NMR spectra of the CDPK regulatory apparatus. HSQC of JC in the absence (A) and presence (B) of Ca\(^{2+}\). Spectra were based on NMR analysis (see below). The same relative affinities are observed for the N- and C-lobes of CaM.

**Fig. 4.** Measurements of Ca\(^{2+}\) affinity by chelator competition method. Plot of calcium occupancy against free calcium concentration for JC (filled circles) and CaM-LD (open circles). Lines drawn are calculated from the best-fit binding constants given in Table I. Error bars shown on the Ca\(^{2+}\) concentration correspond to the uncertainty in the absorption measurement of 0.003 absorbance units.

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participating in Ca\(^{2+}\) signal transduction pathways and points to the great importance of the J region in the CDPK regulatory apparatus.

The C-lobe Contains the Higher Affinity Ca\(^{2+}\) Sites—To learn more about the mode of action of the CaM-LD, NMR was used to characterize the Ca\(^{2+}\) binding events in a site-specific manner. For these experiments, Ca\(^{2+}\) was titrated into a solution of JC, and two-dimensional \(^{1}H,^{15}N\) HSQC spectra were recorded at each step. The intensities of 45 well-resolved residues scattered across the protein sequence could be monitored in these spectra.

At Ca\(^{2+}\) levels below 0.1 mol eq there is severe attenuation of already weak signals from apoJC, presumably because of conformational exchange between the apo and various subsaturated Ca\(^{2+}\) states. Above 0.1 mol eq of Ca\(^{2+}\), a number of signals from the C-lobe begin to appear at the same positions as in the spectrum of fully Ca\(^{2+}\)-loaded JC (Fig. 3B). These signals continue to increase in intensity throughout the Ca\(^{2+}\) titration. The observation of this subset of relatively narrow signals implies the species to which it corresponds is in slow exchange with the heterogeneous ensemble of other states that we presume have partial loading of Ca\(^{2+}\). The putative complete filling of sites at substoichiometric levels of Ca\(^{2+}\) suggests binding occurs in a cooperative manner.

As noted above, the first signals to appear are all from the C-lobe. In fact, differences in the relative intensity of signals from the N- and C-lobes were observed over the full course of the titration. Fig. 5 shows selected results for residues from the four calcium binding loops and for Leu-441 in the J region. At Ca\(^{2+}\) concentrations below 0.5 mol eq, only resonances from residues in the C-lobe (Ala-530 and onward) are observed, and these continue to have significantly higher intensity until the latter stages of the titration. These observations indicate that the high affinity Ca\(^{2+}\) sites are located in the C-lobe. Because the affinities differ by a factor of 25, the assignment of two pairs of binding constants to the individual CaM-LD domains is reliable.

The Junction Region Interacts Predominantly with the C-lobe—NMR was also used to structurally characterize the in-
interaction of J with the CaM-LD. Titration of the J peptide into a solution of CaM-LD caused significant perturbations in a number of CaM-LD resonances. Interestingly, plots of the chemical shift changes of backbone amides against the sequence (Fig. 6) show large changes (≥0.1 ppm) occur almost exclusively for residues in the C-terminal lobe, with the most significant effects on residues in and around helix 7. This observation implies that the binding interactions of J with the C-lobe are stronger. Additional insight was obtained from a qualitative assessment of the rates of amide proton exchange (Fig. 6). When a sample of Ca²⁺-loaded JC was lyophilized then dissolved in D₂O, the vast majority of signals remaining in the ¹H,¹⁵N HSQC spectrum arise from the C-lobe, indicating more protection of this domain. The data are, thus, consistent with the J region interacting predominantly with and stabilizing the C-lobe of the CaM-LD.

One additional point of interest is that the NMR chemical shifts at the end of the titration of J into Ca²⁺-saturated CaM-LD (Fig. 6A) were not all identical to those of Ca²⁺-saturated JC (Fig. 6B), although the overall trend to a greater effect on the C-lobe than the N-lobe is found in both systems. Some of the differences between the unimolecular JC and bimolecular J+CaM-LD systems can be attributed to the absence of the covalent link between J and the first helix in the N-lobe in the bimolecular systems. In addition the sensitivity of amide resonances to the inevitable differences in the experimental conditions must be considered. However, the observed differences extend beyond what is expected from these factors. The most striking aspect in comparing the data in Figs. 6, A and B, is the larger perturbations in the N-lobe of JC relative to J+CaM-LD. These observations suggest that, although the bimolecular system is an excellent model for studying CDPK, the linking of J to the CaM-LD plays a more significant role than simply tethering together two independent regions of the protein.

DISCUSSION

Our biophysical analysis of the regulatory domain in a plant CDPK revealed evidence for two distinctive features. First, the Ca²⁺ binding properties and conformation of the CaM-LD are
dramatically altered by the presence of a covalently tethered J region; for the JC protein, the C-lobe calcium affinity is 5.6 nm, 100-fold stronger than that of the isolated CaM-LD. Second, the functional properties of the N- and C-lobes of the CaM-LD are not equivalent, with measured Ca\(^{2+}\) binding affinities for the C-lobe ~20-fold stronger than for the N-lobe (Table I). Distinct binding constants (0.6 and 55 \(\mu\)M) have also been observed in a tomato CDPK isoform (28). These affinities are significantly lower than those determined for the isolated constructs, although again there is a large affinity difference (~90-fold) between the two lobes of the tomato CaM-LD. Variability in calcium sensitivity has been shown for soybean CDPK, where overall calcium affinities ranged from 51 to 1.6 \(\mu\)M for three isoforms. Furthermore, the presence of different substrates caused varying increases (~80-fold) from these values. In the present case, the high affinity observed for the C-lobe suggests that it may be loaded at basal cytosolic levels of Ca\(^{2+}\), whereas the weaker affinity of the N-lobe suggests that it can respond to Ca\(^{2+}\) fluxes expected during Ca\(^{2+}\) signaling. The following discussion elaborates on these features.

**Are J and CaM-LD Pre-associated at the Basal Level of Ca\(^{2+}\)?—** The observation that the C-lobe has a high Ca\(^{2+}\) affinity when the CaM-LD is tethered to a J region suggests that the CaM-LD and J form a stable interaction at basal Ca\(^{2+}\) concentrations. At such low levels of Ca\(^{2+}\), only the resonances of residues in the C-lobe of JC are visible (Fig. 5), which suggests that the interaction involves the C-lobe of the CaM-LD and the J region. Indeed the C-lobe appears to have stronger interactions with J at all levels of Ca\(^{2+}\) loading (Fig. 6).

For deeper insight into the mechanism of action of the CaM-LD regulatory apparatus, the macroscopic binding constants for CaM-LD and JC were used to simulate\(^3\) the overall Ca\(^{2+}\) loading and the populations of molecules with 1, 2, 3, or 4 ions bound \((p_1, p_2, p_3, p_4)\) (Supplemental Fig. S1). Fig. 7 shows a plot of the populations of the various states of Ca\(^{2+}\) occupancy as a function of the free Ca\(^{2+}\) level based on the measured binding constants. The dissociation constant for the C-lobe is far below the values found for typical Ca\(^{2+}\) sensors, indicating it would be saturated at the basal level of cytosolic Ca\(^{2+}\) (~0.1 \(\mu\)M). This would imply the C-lobe is pre-associated with the J before Ca\(^{2+}\) activation.

![](image)

The values of 0.006 and 0.1 \(\mu\)M measured for the C- and N-lobes of CDPK both fall below the ~1 \(\mu\)M range of a typical Ca\(^{2+}\) sensor. However, it is important to note that these \(K_d\) measurements were made at low ionic strength. Increases in \(K_d\) as ionic strength is increased are well documented, and for CaM, an increase of about a factor of four has been reported when going from 25 to 100 mM KCl (17). Thus, under physiological conditions, the actual \(K_d\) values are expected to be at least 5 times higher, bringing the values to >0.03 and 0.6 \(\mu\)M, fully consistent with pre-association of the C-lobe and assignment of Ca\(^{2+}\) sensor activity for the N-lobe.

Such pre-association of a Ca\(^{2+}\) sensor protein and its target may be more prevalent than initially anticipated. Small angle X-ray scattering experiments show that CaM interacts with skeletal muscle myosin light chain kinase at substoichiometric Ca\(^{2+}\) levels (29). It has long been known that the C-lobe of troponin C (30), which regulates troponin I in a Ca\(^{2+}\)-dependent manner, has a Ca\(^{2+}\) affinity 2 orders of magnitude greater than the N-lobe and is constitutively bound. In this case, the C-terminal lobe anchors the sensor to its target, and the N-terminal lobe serves as the \(\alpha\) regulatory element, as we propose here for CDPK.

The Ca\(^{2+}\) binding properties reveal that the mechanism of Ca\(^{2+}\) activation of CDPK is clearly distinct from CaMKs. Our data imply that the Ca\(^{2+}\)-dependent response of the CaM-LD is triggered by the binding of Ca\(^{2+}\) to the N-lobe. The differences in Ca\(^{2+}\) affinity between the two EF-hand regulatory domains in CDPK are significantly larger than the differences observed between the domains of CaM in the complex with myosin light chain kinase (Table I). For CaM, the transition from 0 to 4 Ca\(^{2+}\) ions bound occurs over a narrow Ca\(^{2+}\) concentration range (Table I), and all binding events appear to contribute to Ca\(^{2+}\)-dependent activation of the kinase. In contrast, binding to the N-lobe of CDPK alone seems likely to serve as the trigger for activation of the kinase.

**Interdomain Communication between the N-lobe and C-lobe of the CaM-LD—** The NMR analysis of the regulatory apparatus provides detailed information about the coupling of the various Ca\(^{2+}\) and J binding events. For example, in all NMR spectra at Ca\(^{2+}\) levels above 0.1 mol eq, the only resonances...
observed correspond to fully Ca\(^{2+}\)-loaded JC. Although this is due in part to the absence of peaks as a result of exchange broadening, the data strongly support cooperative binding of Ca\(^{2+}\). Consider the intensities of the resonances of three residues, Ile-471, Ile-543, Ile-577, which occupy sites in the Ca\(^{2+}\) binding loops that are known to be highly sensitive probes of Ca\(^{2+}\) binding (31). The similarity in the intensity profiles for the C-lobe pair (Ile-543 and Ile-577) (Fig. 5) agrees well with the cooperative Ca\(^{2+}\) binding in the C-lobe, whereas the slower response of Ile-471 in the N-lobe is consistent with a response to Ca\(^{2+}\) binding events in a lower affinity domain. Leu-441 in the J region correlates well with the C-lobe residues, consistent with association of J upon binding of Ca\(^{2+}\) to the C-lobe.

Analysis of the populations of different Ca\(^{2+}\)-bound states based on the simulations noted above indicate that the peak intensity for resonances in the N-lobe should be negligible until the Ca\(^{2+}\) levels are sufficiently high to mostly saturate the C-lobe, i.e. at $-1.6 \text{ mol eq of Ca}^{2+}$. However, for the majority of the N-lobe, resonances begin to appear at $-0.4 \text{ mol eq of Ca}^{2+}$. These data strongly suggest additional levels of coupling of the Ca\(^{2+}\) and J binding events.

One possible explanation for this observation is that the N-lobe may populate the open conformation of the Ca\(^{2+}\)-saturated state before Ca\(^{2+}\) is bound. In this context it is important to consider that the open conformation with exposed hydrophobic surface will be stabilized when J is bound. Moreover, once J is engaged by the C-lobe, the local concentration of J in the vicinity of the N-lobe is very high. A calculation using a simplified model gives an estimate of the maximum possible effective concentration of J equivalent to $-6 \times 10^9 \text{ M free junction peptide}$. Thus, although there is a very small equilibrium population of the N-lobe open state resulting from binding of Ca\(^{2+}\), the high concentration of J could trap this conformation. As a consequence, the N-lobe residues could exhibit chemical shifts consistent with the fully engaged JC complex even though the equilibrium population of the Ca\(^{2+}\)-saturated N-lobe state is very small. Our results strongly imply that interdomain communication between the C- and N-lobes of CDPK is mediated through interactions with the adjacent J region.

**Activation of CDPKs Differ From a Typical Calmodulin-dependent Protein Kinase**—As expected, there are many similarities between the structures and properties of the CaM-LD and CaM. First, as in CDPK, CaM interacts with a CaM binding segment at the C terminus of the catalytic domain of several kinases (32). Second, diffusion NMR studies on the bimolecular complex of soybean CaM-LD in complex with a J peptide (33) and corresponding small angle X-ray scattering studies with the *Arabidopsis* JC used in this work indicate the Ca\(^{2+}\)-saturated state of JC has a compact structure like CaM target complexes. Third, the NMR (Fig. 1b) and CD (Fig. 2) analyses presented here reveal similarity in structure. Fourth, CaM can activate CDPKs in truncation mutants lacking the CaM-LD, i.e. analogous to a calmodulin-dependent protein kinase (although never to a fully activated state) (8). Fifth, there is precedent for CaM interacting with targets predominantly via the C-lobe, e.g. the plasma-membrane pump (34).

Despite these many similarities, our experiments indicate that the CaM-LD has several distinct features that result in a unique mode of action. Among these are the covalent attachment of the J region and the large difference in Ca\(^{2+}\) affinities between the N-lobe and the C-lobe. These greatly alter the manner in which the CaM-LD interacts with J relative to how CaM is anticipated to interact with its targets. The high local concentration due to covalent attachment of the CaM-LD and J implies that the binding properties of the CaM-LD are finely tuned to enable it to serve in a regulatory capacity. The C-lobe of the CaM-LD makes the predominant interactions with J, and the flexible attachment of the two lobes of the CaM-LD by a short linker provides a means for coupling their independent activities. In fact, our studies reveal a significant degree of coordinated action of the two lobes of the CaM-LD. The measurement of the Ca\(^{2+}\) and target binding affinities has, therefore, elucidated some of the key subtleties associated with the interaction with the autoinhibitory J region and activation of the kinase.
Implications for the Mechanism of the Regulatory Apparatus—The biophysical and structural analyses presented here indicate that the CaM-like regulatory apparatus of CDPK undergoes multiple structural transitions. A model for CDPK activation, which summarizes the biophysical data described in this work, is shown in Fig. 8. The model uses the difference in affinity between the two domains to show separate functions for the lobes; at basal levels of Ca\(^{2+}\) in the cell, the C-lobe of CDPK is Ca\(^{2+}\)-bound and associated with the J region, whereas the N-lobe is free to respond to intracellular Ca\(^{2+}\) signals and, thus, serves as the critical Ca\(^{2+}\)-dependent regulatory element. A Ca\(^{2+}\) sensor assignment for the N-lobe is consistent with the studies described above on a tomato CDPK and mutational studies conducted on a CDPK from \textit{P. falciparum} (pf-CPK1) \(4\), where activation and a Ca\(^{2+}\)-dependent alteration in conformation were both abolished by mutations that disrupt Ca\(^{2+}\) binding residues in EF hands 1 and 2. In contrast, similar mutations that disrupted the C-lobe EF-hands had only minor effects on activation.

Although the C-lobe may not function directly as the Ca\(^{2+}\) sensor, the two EF-hands are nevertheless highly conserved in most CDPKs. It is anticipated that the pre-association of J with the C-lobe is a general feature of CDPKs. It is conceivable that this interaction is a means to stabilize the regulatory apparatus by reducing the population of the conformationally heterogeneous Ca\(^{2+}\) free state.

One obvious limitation of our studies is the characterization of Ca\(^{2+}\)-dependent properties of the regulatory apparatus in isolation from the kinase domain it regulates. Ideally, the Ca\(^{2+}\) affinities need to be measured for the intact protein and correlated with experiments to determine Ca\(^{2+}\) thresholds for kinase activation. However, the analysis of such a complex system at a fundamental molecular level would be extremely complicated, particularly since there are no structures available of any Ca\(^{2+}\) regulatory element bound to a kinase. The approach we and others have taken is to attack the problem by characterizing smaller fragments, with the goal of building up the whole from the parts.

In analyzing the binding data for CDPK, it is essential to consider that the interaction of the J region with the kinase has the potential to compete with the binding between the J region and the CaM-LD. Competition in the context of the holoenzyme will weaken the Ca\(^{2+}\) affinities determined here for an isolated regulatory domain. In fact, as detailed above, lower Ca\(^{2+}\) affinity than we have measured would not compromise the ability of the CaM-LD to serve as a Ca\(^{2+}\) sensor.

A second critical consideration is that Ca\(^{2+}\) activation thresholds of kinases are actually fairly variable. The clear dependence on different peptide substrates shows there is a strong coupling between the peptide substrate, catalytic domain, the junction region, and the CaM-LD. For example, with soybean CDPK isoform a, the lowest Ca\(^{2+}\) concentration observed for half-maximal (\(K_{0.5}\)) activity was 60 nM, using syntide-2 as a substrate (35). The activation threshold for this same isoform increased by 2 orders of magnitude (i.e. 6 \(\mu\)M) when provided histone as a substrate. Given the potential of interconnecting domains to alter the activation threshold, our analysis of the isolated JC represents the foundation upon which other studies of Ca\(^{2+}\)-dependent regulation of CDPK can be built.

In addressing the functional role of plant CDPKs, it is important to ask why so many isoforms co-exist in a single species (34 in the \textit{Arabidopsis} genus). A potential explanation is that different isoforms have co-evolved with biochemically distinct CaM-LDs that provide different Ca\(^{2+}\) activation thresholds (1). Different Ca\(^{2+}\) activation thresholds have been reported for different isoforms. Some CDPK isoforms have one or more divergent EF hands, with eight \textit{Arabidopsis} CDPK-related kinases having all four EF-hands predicted to be non-Ca\(^{2+}\) binding. In considering the diversity of CDPKs and related kinases, the biophysical analysis presented here emphasizes the likely role of the differing function of the two lobes of the CaM-LD.

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Evidence for Differing Roles for Each Lobe of the Calmodulin-like Domain in a Calcium-dependent Protein Kinase

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