Unexpected Structure of the Ca\textsuperscript{2+}-regulatory Region from Soybean Calcium-dependent Protein Kinase-α

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Calcium-dependent protein kinases (CDPKs) are an extensive class of multidomain Ca\textsuperscript{2+}-regulated enzymes from plants and protozoa. In vivo the so-called calmodulin-like domain (CLD) of CDPK binds intramolecularly to the junction domain (JD), which exhibits both kinase-inhibitory and CLD binding properties. Here we report the high resolution solution structure of the calcium-regulatory region from soybean CDPK-α determined in the presence of a peptide encompassing the JD. The structure of both lobes of CLD resembles that of related helix-loop-helix Ca\textsuperscript{2+}-binding proteins. NMR chemical shift mapping studies demonstrate that the JD induces significant structural changes in isolated Ca\textsuperscript{2+}-CLD, particularly the C-terminal domain, although a stable complex is not formed. A CLD solution structure calculated on the basis of NMR data and long range fluorescence resonance energy transfer distances reveals an activated state with both lobes positioned side by side, similar to calcineurin B rather than calmodulin, highlighting the possible pitfall of assigning function purely from sequence information.

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**MATERIALS AND METHODS**

**Protein and Peptides**—The gene for CLD from soybean CDPK-α (designated ΔCaL15N-Lys508) was a kind gift from Dr. A. C. Harmon (University of Florida). The CLD gene was PCR-amplified out of the original pET-3b construct and cloned into the pT7-7 vector, and a His tag was inserted immediately C-terminal to the protein-expressing sequence to avoid purifying proteins subject to proteolytic cleavage as reported previously (10). *Escherichia coli* BL21(DE3) cells harboring this construct were used as hosts for protein expression. 15N-Labeled CLD was expressed using a MOPS-based minimal medium as described previously (18), and 15N/13C-labeled CLD was expressed using Bio-Express minimal medium (Cambridge Isotope Laboratories). All purification steps were carried out at 4 °C. Cells were resuspended in binding buffer (50 mM HEPES, 0.5 mM NaCl, 5 mM imidazole, 2 mM CaCl₂, pH 7.8) and an N-terminal nickel-loaded metal chromatography resin (Amer- sham Biosciences). Wash buffer (50 mM HEPES, 0.5 mM NaCl, 25 mM imidazole, 2 mM CaCl₂, pH 7.8) was applied until the A280 of the flow-through stabilized. Elution buffer (50 mM HEPES, 0.5 mM NaCl, 500 mM imidazole, 2 mM CaCl₂, pH 7.8) was added, and the protein was collected for further purification. Subsequently calcium-dependent hydrophobic chromatography was carried out using Sepharose 4B (Amer- sham Biosciences) as described previously (14). Purity was assessed by SDS-PAGE followed by Coomassie Blue staining. Protein samples were desalted into a 5 mM ammonium bicarbonate solution using a PD-10 column (Bio-Rad) and subsequently lyophilized and stored at −20 °C prior to being used in NMR experiments. Unlabeled peptide encompassing the CLD-binding portion of the junction domain of soybean CDPK-α and an N-terminal CLD peptide in 350 mM triethanol, 10 mM CaCl₂, pH 7.2, pH was adjusted with KOD and DCl, and the final pH was not adjusted for isotope effects. 13N samples used for Ca²⁺ and initial JD-CLD titrations were 150–200 μM in a 500-μL volume, and the final sample used for resonance assignment of Ca²⁺-CLD was 1 mM in protein. The JD-CLD 13N/13C samples used for assignment and structural determination was ~1 mM in protein and 1.2 mM in JD peptide in 350 μL. 1H/15N/13C backbone resonance assignments of the CLD in the JD-CLD sample were accomplished with standard triple resonance experiments (19, 20) on a 500-MHz Bruker Avance DRX spectrometer equipped with a 5-mm TXI cryoprobe with a 2-axis gradient channel. All spectra were referenced with respect to a 1H chemical shift of 0 ppm for the most upfield resonance of 5,5-dimethylsiline and 8.0 ppm for 15N-norleucine. Residual dipolar coupling data were obtained from the same sample diluted to 500 μL with the inclusion of a pentaethylene glycol monododecyl ether/l-hexanol-based detergent system (50, r = 0.89) as described previously (21) using an in-phase-antiphase 1H,13N HSQC pulse program on a Bruker Avance DRX 700 spectrometer equipped with a 5-mm TBI probe with triple axis gradients. Most experiments in the liquid crystalline phase were acquired at 36 °C, and measurements in the isotropic phase were acquired at 39 °C. All data were processed with NMRPipe (22) and analyzed with NMRView (23). A three-dimensional 13N-edited NOESY spectrum (24) was also acquired on the Ca²⁺-CLD to confirm 1H,13N HSQC correlations by chemical shift mapping of JD-CLD interactions. The degree of chemical shift movement was assessed using the equation,

$$\Delta J_{obs} = \Delta J_{calc} + \Delta J_{Theory}$$

(Eq. 1)

where ΔJ is the difference in hertz between the Ca²⁺-CLD and the JD-CLD.

**Structure Determination**—Side chain aromatic and structural NOE data for the CLD protein were obtained from a 1H/15N/13C simultaneous NOESY (25) obtained at 800 MHz on a Varian Inova spectrometer (Canadian National High Field NMR Centre, University of Alberta) with a mixing time of 150 ms. Attempts to obtain intermolecular NOEs (26) between protein and peptide were unsuccessful at 500, 600, and 700 MHz using both Bruker and Varian pulse sequences as were isotope-filtered experiments (27) to obtain structural information about the JD peptide alone. ARIA 1.2/CNS 1.1 was used for all structure calculations (28). Dihedral angle restraints were derived using both CSI (29) and TALOS (30) based on analysis of the chemical shifts. Other non-Gly residues without these constraints were limited to dihedral angles of −35° to −175° based on accepted geometry. The simulated annealing protocol was initiated from a fully extended conformation.

The default parameters were used for ARIA with the exception that...
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RESULTS

Binding of JD Peptide Causes Significant Structural Changes to the CLD—Expression and purification of soybean CLD was readily achieved from both rich medium and isotonically enriched minimal medium, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed a single protein band near the expected molecular weight. Initial $^1$H, $^{15}$N HSQC correlation spectra of both Ca$^{2+}$-saturated CLD (Ca$^{2+}$-CLD) and Ca$^{2+}$-CLD with JD peptide encompassing residues 301–329 of soybean CDPK-α peptide added (JD-CLD) demonstrated reasonable peak dispersion indicative of a folded protein (data not shown). There was, however, significant variation in peak intensities (Fig. 2A for JD-CLD) and fewer than the expected 180 resonances suggesting complex conformational behavior with many broad peaks and some split into multiple dispersive resonances, such as resonances 369 and 477 in Fig. 1, B and C. This appears to be characteristic of CDPKs as complete resonance assignment of the regulatory region of an Arabidopsis isoform (inhbititory and calcium-binding regions together in the same construct) required low pH and significantly elevated temperatures to overcome resonance broadening (32). Significant chemical shift differences were noted between the Ca$^{2+}$-CLD and Ca$^{2+}$-saturated JD-CLD spectra due to the presence of the JD peptide (Fig. 2C) as discussed below. Structure determination proceeded with the JD-CLD spectra where residues from the C-terminal lobe of the CLD were generally better defined (i.e. more intense and less broad than in the Ca$^{2+}$-CLD sample), although the N- and C-terminal domains still demonstrated significantly different chemical properties. For example, the backbone resonances were generally broader and less intense in the C-terminal domain in both the $^1$H-$^{15}$N HSQC correlation spectra (Fig. 2A) and $^{13}$C/$^{15}$N-edited NOESY spectra (not shown), and these C-domain correlation peaks also demonstrated a greater distribution of $^{15}$N-$^1$H NOE values (Fig. 2B). Interestingly in both domains the peak intensity was highest near the loop regions of the helix-loop-helix metal-binding sites, but in the N-terminal domain the $^{15}$N-$^1$H NOE values were fairly constant, suggesting the difference in intensity is not caused by differential flexibility.

Essentially complete chemical shift assignments were obtained for residues in the Ca$^{2+}$-binding loops as well as the linkers between calcium-binding loops 1 and 2 (N-terminal domain) and loops 3 and 4 (C-terminal domain). Partial assignments were obtained for residues Ala$^{329}$–Glu$^{335}$ (tether region at the N terminus of the CLD), Lys$^{405}$–Leu$^{412}$, the linker between the two domains, and Arg$^{485}$–Leu$^{496}$ a stretch of the C-terminal tail. The 14 extreme C-terminal tail residues that were assigned showed very narrow line widths, representative of rapid motions, presumably due to a flexible random coil conformation. No significant structural NOEs were observed for this region, and hence only residues Ala$^{329}$–Arg$^{485}$ were included in the structural calculation.

The structures of the N-terminal and C-terminal domains of CLD conform to bilobal EF-hand proteins with a canonical helix-loop-helix structure for the two calcium-binding loops in each domain and a small β sheet region connecting the pairs (Fig. 3A). When the core regions of the N- and C-terminal domains of the calcium-regulatory region are overlaid independently, they are well defined (backbone r.m.s.d. values of 0.780 and 0.604 Å, respectively, Table I). A significant number of long range NOEs, particularly from the $^{13}$C-edited NOESY, define the hydrophobic centers in each domain. The relative spatial positioning of the two domains with respect to each other is discussed below. Table I provides summary statistical information for the family of 15 lowest energy structures from the ARIA calculations. In the N-terminal domain, these hydrophobic cores are flanked by interhelical contacts between helices A/B, A/D, B/C, and C/D (helices as defined in Fig. 1A). Correspondingly, in the C-terminal domain, a similar pattern of helical interaction is seen with contacts between helices E/F, E/H, F/G, and G/H.

The interhelical angles in the helix-loop-helix Ca$^{2+}$-binding loops (Table II) are all slightly more closed than a perpendicular value of 90°. The most closed is the first A/B EF-hand at 116 ± 4°, while the C/D, D/E, and E/F helix angles show similar values of 103 ± 6, 103 ± 4, and 102 ± 4°, respectively. These helical angles result in pockets of hydrophobic surface area being exposed in both domains. Residues Leu$^{443}$, Leu$^{447}$, Leu$^{471}$, Thr$^{475}$, Cys$^{478}$, and to a lesser extent Ile$^{497}$, Ile$^{500}$, and Phe$^{505}$ contribute to a significant pocket in the N-terminal domain. In the C-terminal domain, major contributions come from Tyr$^{418}$, Phe$^{419}$, Phe$^{436}$, Phe$^{439}$, Leu$^{441}$, Ile$^{444}$, Met$^{469}$, and Met$^{470}$, and minor contributions come from Ile$^{466}$ and Phe$^{466}$. It is worth noting that the Met residues of the N-terminal domain (residues 346, 372, and 380) are all located on the outside surface or interior of the lobe and do not con-
Fig. 3. A, ensemble of the 15 lowest energy structures for the N-terminal domain (backbone r.m.s.d., 0.78 Å) and C-terminal domain (backbone r.m.s.d., 0.60 Å) of the Ca²⁺-saturated JD-CLD molecule. The disordered region in red is the tether region immediately N-terminal (N-term) to the well ordered region, and in intact CDPK, the JD is attached directly N-terminal to this region. Analogously the green C-terminal (C-term) tail is disordered, although several contacts are made between the C-terminal domain and the C-terminal tail. B, stereo ribbon diagram of the open conformation of Ca²⁺-saturated JD-CLD where the two domains are adjacent positioned forming an open surface. C, chemical shift mapping changes between the Ca²⁺-CLD form and Ca²⁺-saturated JD-CLD form of the N- and C-terminal domains. Changes in the chemical shift are marked for amide ¹H,¹⁵N changes of <50 Hz (blue), 50–100 Hz (cyan), 100–150 Hz (yellow), and >150 Hz (red) as described in the text. Figures were generated with MolMol (58).
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The hydrophobic pockets in both domains are lined by a significant number of charged residues. In the N-terminal domain, the side chains of Glu370, Glu375, and Asp376 form a cluster of negative charge. The C-terminal lobe does not show a similar pattern of clustering with only Asp383 and His387 protruding significantly into the hydrophobic pocket, while Asp384 and Glu462 are situated at the periphery. It is interesting to note that the region between Asp441 and Asp448 show extensive broadening on JD binding. For example, the intensity of the ring protons of Tyr418 (helix E) also become increasingly broadened on JD binding. For example, the intensity of the ring protons of Tyr418 (helix E) also becomes increasingly broadened on JD binding. The opposite trend was observed for selected residues that showed sterically unambiguous contacts with the side chain of Lys427 (also helix C). This corresponds roughly to the ends of helix C and the beginning of helix E (Fig. 1B). The intensity of the observed interdomain NOEs was very weak presumably because the resonances for the residues involved, especially Ala382, exhibited several distinct conformers. Nevertheless the interaction between the two lobes is primarily comprised of a hydrophobic interaction with Tyr418 forming the primary bridge.

While the interface between the two lobes could not be defined precisely due to exchange broadening of some of the residues involved in the interaction, the interdomain NOEs and residual dipolar coupling (RDC) measurements allowed for the elucidation of approximate global orientations of the two domains. When the full family of 15 structures is overlaid, the backbone r.m.s.d. is 5.5 Å for the well defined regions. However, further analysis of the structures shows two distinct subpopulations. In the first set, the two domains are positioned side by side, forming an elongated hydrophobic face (Fig. 3B) or “open” state. The r.m.s.d. for the four most similar structures in this family is 2.07 Å. In the other extreme, the N-terminal and C-terminal lobes sit opposite to each other in a “closed” state (not shown). When the most similar six structures with this orientation are fit, the backbone r.m.s.d. drops to 1.32 Å. The remaining five structures are in orientations in between these two extremes. In all structures, there are interactions between helix C from the N-terminal domain and helix E from the C-terminal domain, forming the basis of a “hingelike” motion centered in this region. Combined with information from long range FRET distance measurements, the open conformation is examined in more detail under “Discussion.”

The C-terminal Domain of CLD Adapts to the JD Peptide—The bimolecular experimental approach used in this study allowed us to further investigate the conformational changes associated with binding of JD peptide to Ca$^{2+}$-CLD to create the Ca$^{2+}$-saturated JD-CLD molecule. Using a chemical shift mapping approach (33), it is readily apparent that the largest changes in chemical shift, and hence structure, are associated with the C-terminal domain (Fig. 3C). In particular, significant shifts are associated with the E and F helices (residues 412–419 and 432–437, respectively) in the transition from Ca$^{2+}$-CLD to Ca$^{2+}$-saturated JD-CLD as observed for all nuclei in both $^1$H, $^1$H HSQC and $^{15}$N-edited NOESY spectra. From the structure, it is apparent that this region forms an integral region in the binding of the JD peptide. Moreover it is noteworthy that several residues in helix F and the linker between Ca$^{2+}$-binding loops 3 and 4 of the C-terminal domain were either not detected from the Ca$^{2+}$-CLD sample or showed extensive broadening; however, they gave relatively strong signals in the JD-CLD sample. This observation suggests that the presence of the JD acts to stabilize portions of the C-terminal domain and “lock” it into a more conformationally limited space, although we emphasize that it is not necessarily completely stable as indicated by the $^{15}$N-$^1$H NOE data (Fig. 2B) and that the two domains are still likely conformationally distinct.

The opposite trend was observed for selected residues that become increasingly broadened on JD binding. For example, Ser414 is essentially completely broadened out in the JD-CLD sample, whereas it is readily observed in the Ca$^{2+}$-CLD sample. Structurally the side chain is situated directly on the peptide binding face essentially in the middle of the hydrophobic cleft. Significant exchange broadening and lower peak intensity (Fig. 2A) are also observed for residues near and within the linker between EF-hand loops 1 and 2 of the N-terminal domain, particularly residues 371 and 375. Within the N-terminal domain, this region (especially residues 365 and 368) is the most affected in the chemical shift mapping studies (Fig. 2C), suggesting that this region of the C-terminal domain interacts most closely with the JD peptide. As a result, we

Table I: Statistics for the 15 lowest energy JD-CLD structures

| r.m.s.d. from distance restraints (Å) | 0.083 ± 0.011 |
| r.m.s.d. from dihedral restraints (Å) | 0.571 ± 0.065 |
| Deviations from idealized geometry |
| Bonds (Å) | 0.003 ± 0.0002 |
| Angles (°) | 0.556 ± 0.014 |
| Impropers (°) | 0.505 ± 0.020 |
| PROCHECK Ramachandran analysis (%) |
| Most favored regions | 69.7 |
| Additional allowed regions | 27.9 |
| Generously allowed regions | 2.7 |
| Disallowed regions | 0.0 |
| van der Waals energy (kcal/mol) | 272 ± 12 |
| r.m.s.d. from lowest energy structure (Å) |
| Residues 338–404 (N-terminal domain) | 0.780 |
| Backbone | 1.129 |
| Residues 412–470 (C-terminal domain) | 0.604 |
| Backbone | 1.112 |
| Heavy | 5.613 |
| Heavy | 5.585 |
| Well defined regions (N- and C-domains) |
| Backbone | 3051 |
| Intraresidue | 1106 |
| Sequential | 692 |
| Medium range | 510 |
| Long range | 743 |
| Ambiguous (total) | 1183 |
| Other restraints |
| Ambiguous (total) | 1183 |
| Other restraints |
| Other restraints |
| H-N residual dipolar coupling | 122, 122 |
| H-N residual dipolar coupling | 45 |

Note: the overall orientation of the two domains is better described by two subsets as described in the text.
conclude that the N-terminal domain and in particular residues at the end of helices A and B, in the linker between two calcium-binding regions, and throughout helix C (due to the hinge-like effect interaction with the C-terminal domain) are also involved in the JD-CLD interaction perhaps via the tether region.

In addition to the chemical shift mapping and resonance broadening observations (Fig. 2, A and C), RDC data suggest that the C-terminal domain plays a greater role in the JD-CLD interaction. In the C-terminal domain, the dispersion of RDCs was 17.0 Hz (+8.3 to −8.7), while in the N-terminal domain it was only 9.2 Hz (+4.4 to −4.8) despite the fact that there were significantly more RDCs measured in the N-terminal domain (17 versus 28). Recent work characterizing RDCs suggests that such patterns are characteristic of conformational heterogeneity (34); hence the lower dispersion in RDCs measured from the N-terminal domain is likely the result of conformational averaging. This is a clear indication that in the presence of the JD peptide, the N-terminal domain is not affected to the same extent as the C-terminal domain especially given the lack of perturbation in chemical shift (Fig. 2C).

**DISCUSSION**

The calcium regulatory properties of CDPKs are critically important for the fine tuning of kinase function. For example, the activation properties of three soybean CDPK isoforms have been shown to be exquisitely sensitive to calcium concentration, and in turn calcium response has been shown to be sensitive to substrate (35). Considering that one of the smallest plant genomes, *Arabidopsis*, already possesses 34 CDPK genes (13), an understanding of the calcium response is critical to delineating the roles of individual CDPKs. It is apparent that, despite significant sequence homology to CaM, the calcium-regulatory regions from CDPK function in a distinctive manner. CaM is a secondary messenger for numerous signaling proteins, and hence there is little room for variability or mutation. In contrast, each CLD is specifically tuned to the unique needs of the particular CDPK that it regulates, allowing for many more degrees of freedom in co-evolution. The results reported here begin to delineate the manner in which this highly tailored response may be initiated from a structural point of view and how this impacts what is known about the CLD.

**Relative Domain Orientation—**An intriguing question raised by the current study is the nature of the interaction between the N- and C-terminal domains of the CLD. The spatial orientation of the two lobes could not be precisely established based on NMR evidence alone; however, when combined with long range interdomain distances derived from FRET studies (17), a much clearer picture emerges. The open conformation uniquely was selected by the FRET distances, and hence we conclude that this is likely the significant population, acknowledging that the steady-state FRET restraints represent average distances. By extending this analysis and incorporating FRET distance restraints directly into the ARIA calculation as described under “Materials and Methods,” a graphic depiction of the per residue r.m.s.d. demonstrates that the most disordered regions correspond to both the surfaces of the putative binding regions between the JD-CLD and the interdomain linker region (Fig. 4). This region corresponds to areas of greatest chemical shift change before and after binding the JD peptide (Fig. 3C) and regions of lowest intensity peaks (Fig. 3A). We emphasize once again, however, that the addition of FRET restraints provides only an average “snapshot” of the relative orientation of the two domains since the environment is very obviously different for the two as shown in Fig. 2 and implied by the different alignment tensors for the two domains in RDC studies. As a result, we propose a model in which the C-terminal domain remains flexible in the presence of the JD peptide (hence the lower 15N-[1H] NOE values and lower intensities due to exchange broadening; Fig. 2, A and B) but generally sits in the open conformation.

**Comparison with Ca^{2+}-binding Proteins—**A BLAST search (36) of the CLD sequence revealed that, in addition to vertebrate CaM (40% identity), the CLD shares significant homology to other calcium-binding proteins in the Protein Data Bank (37), most significantly to vertebrate skeletal muscle troponin C (sTnC, 35% identity) and human calcineurin B (CNB, 31% identity). All of these proteins have similar primary, secondary, and to a certain extent tertiary structure to JD-CLD binding four equivalents of Ca^{2+} with helix-loop-helix motifs in a bilobal arrangement. When compared with Ca^{2+}-saturated structures, the pattern of interhelical contacts is analogous to that of CaM. In contrast, each CLD is specifically tuned to the unique regulatory regions from CDPK function in a distinctive manner.
seen for Ca\(^{2+}\)-CaM, although the first EF-hand of the CLD appears to be slightly more closed than the recently determined solution structure (38) and significantly more closed than the x-ray structure of Ca\(^{2+}\)-CaM (39) (Table II). Least-squares fitting of the best defined regions of the two domains of CLD and the two lobes of CaM produce r.m.s.d. values between 1.86 and 2.82 Å for the NMR structure and 2.01 and 2.49 Å for the x-ray structure (Table III). It is interesting to note that the N-terminal domain of CLD shows the best fit in both cases but with opposite domains (i.e. the N-terminal lobe of the CaM NMR structure and the C-terminal lobe of the x-ray structure). This may be reflective of the difference in helix angles between the CaM NMR and x-ray structures (38). Similar results are seen with sTnC. While data are not available for the apoCLD form it is reasonable to suggest that the regulatory mechanism may be similar to the function of CaM (40) and troponin C (41). In particular, the exposure of the hydrophobic patches in CLD as described above is a Ca\(^{2+}\)-dependent phenomenon through the Ca\(^{2+}\)-dependent folding of CLD (16). One key difference, however, is noted in the target-bound state with the C-terminal domain of JD-CLD showing significant structural changes as evidenced by our chemical shift mapping studies, while the backbone conformation of the two lobes of CaM remain essentially the same in both the Ca\(^{2+}\) and target complex forms (42).

Another key difference in the structures is the open subfamily observed within the JD-CLD structures. This is surprising based on CaM-CaMKII kinase complex structures in which the two domains envelope the target peptide (42). For this reason, it is particularly noteworthy that CNB was found to share significant identity with the CLD as it is found in a completely open conformation with an elongated hydrophobic cleft spanning the two lobes of the protein in the Ca\(^{2+}\)-saturated form (43) as demonstrated in Fig. 5. The interhelical angles for CNB are reasonably similar to both JD-CLD and CaM (Table II), although the C-terminal domain of CLD shows slightly more resemblance in this case. Interestingly this is not reflected in the backbone superposition where both domains of the CLD fit significantly better to the N-terminal domain of CNB (Table III) likely due to a significant number of extra residues in the linker between EP-hands 3 and 4 of CNB. It is also worth pointing out that, similar to the CLD, CNB has a short N-terminal extension prior to the first Ca\(^{2+}\)-binding loop. In CNB, this extension comes back toward the C-terminal domain and packs in to the linker region between the two domains. In a putative intramolecular binding model of the JD-CLD, the tether region and JD would be required to similarly cross the face of the N-terminal domain. Fig. 4 shows that these regions are most disordered and lie on the surface of the putative interaction surface supporting the notion of concerted action. Furthermore the CNB crystal structure also shows packing interactions between the helices equivalent to C and E of JD-CLD, although they occur at different ends of the helix, and

| Protein Data Bank code | ApoCaM (N) | ApoCaM (C) | Ca\(^{2+}\)-CaM NMR (N) | Ca\(^{2+}\)-CaM NMR (C) | Ca\(^{2+}\)-CaM x-ray (N) | Ca\(^{2+}\)-CaM x-ray (C) | Ca\(^{2+}\)-CNB x-ray (N) | Ca\(^{2+}\)-CNB x-ray (C) |
|-----------------------|------------|------------|-------------------------|-------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| JD-CLD (N terminus)   | 1DMO       | 1DMO       | 1I7O                    | 1I7P                    | 1CLL                     | 1CLL                     | 1TCO                     | 1TCO                     |
| JD-CLD (C terminus)   | 4.29       | 4.11       | 2.82                    | 2.40                    | 2.49                     | 2.35                     | 3.65                     | 3.65                     |

\(^a\) See Table II for references to structures.

TABLE III

r.m.s.d. values between lobes of JD-CLD and lobes of CaM, CNB, and sTnC (Å)

The N-terminal domains are shown in red along the y axis.

closer packing is observed between helices D, E, and F that is not seen with JD-CLD. Finally it is worth noting that yeast CaM has been shown to demonstrate interactions between the N- and C-terminal domains (44, 45) and that molecular dynamic simulations of vertebrate CaM suggest transient interactions between the two lobes confirmed by a recent crystal structure of a collapsed Ca\(^{2+}\)-CaM (46). Also worthy of note is that several calciunin B-like proteins have been found in plants (4). Arabidopsis isoform AtCBL2 has recently been crystallized (47); it shares the same overall fold as human CNB, although it has slightly lower sequence homology to the CLD from this study (~27% for the Ca\(^{2+}\)-binding region). Taken as a whole, it is clear that the CLD shares some structural features of CaM and some of CNB and may use some type of hybrid mechanism because independence of the two domains is not required in vivo.

Pivoting: a Proposed Mechanism—The bimolecular approach used in this study allowed us to examine the transition from a Ca\(^{2+}\)-saturated form of CLD to a state where the influence of the JD was monitored. While presumably the Ca\(^{2+}\)-saturated state does not exist alone in vivo, studying its role allows us to

\(^a\) Shepherd, C. M., and Vogel, H. J. (2004) Biophys. J., in press.
Fig. 6. Schematic diagram of JD-CLD interaction. Structural evidence suggests that in the absence of JD peptide there are multiple conformations of the C-terminal lobe, while NMR diffusion and FRET-derived long range data show that the protein is collapsed although not necessarily into a single stable conformation. In this situation, the tether region also has significant flexibility as depicted by the dashed lines. Subsequently the C-terminal lobe of the CLD interacts most significantly on addition of JD peptide to Ca\(^{2+}\)-CLD as the evidence from the current study indicates. The N-terminal lobe likely acts as a scaffold that allows the tether region to position the JD. In the current study where a bimolecular system was used, the tether retains its flexibility, although presumably in the intact protein it would remain less mobile since it is directly attached to the JD. It is not clear to what extent the tether region, linker region, and flexible regions of the C-terminal lobe of the CLD act in concert on binding the JD.

We have provided strong evidence that the interaction between the C-terminal lobe of the CLD and the JD is critical from a structural point of view (Figs. 3C and 4). A similar conclusion, from a functional point of view, was reached by a study of soybean CDPK-α (10) in which the full-length protein was truncated at the C terminus to exclude the last two calcium-binding sites and the linker region. Activity was partially restored by addition of exogenous CLD; however, exogenous CaM had little effect. This result was surprising because a chimeric protein in which the CLD is replaced by CaM results in an active kinase. The results of the current study demonstrate that the structural mechanisms by which CaM and CLD act are quite different, and the binding cavity presented to the JD is likely unique for either molecule. It appears that either one mechanism or the other is able to activate the CDPK, but a combined mechanism is not effective.

The strong contribution of the C-terminal domain to binding is also interesting given the interpretation of results from previous experiments characterizing the role of individual EF-hands to activation. Work with Plasmodium CDPK has shown that mutations in the calcium-binding loops of the N-terminal domain are detrimental to activation, whereas mutations in the C-terminal domain result in minor effects (51). Based on the structure presented in this work, we would suggest that, in the presence of the JD, either the ability or necessity of the C-terminal lobe to bind Ca\(^{2+}\) changes, and hence mutations in the C-terminal lobe have less effect than in the N-terminal domain. A similar conclusion has been reached in the case of CaM/myosin light chain kinase where the effectiveness of similar mutations on Ca\(^{2+}\) binding are dependent on the presence of binding targets (52). In the case of CLD, if the N-terminal domain were acting solely in a structural capacity allowing the JD and C-terminal domain to approach each other, it would be critical for Ca\(^{2+}\) to bind, and the mutations would be detrimental. However, once the JD was near the C-terminal domain in a high local concentration, the mutations in the EF-hands would not be as critical to the structure especially given the “adaptation” of the C-terminal domain to the JD.

While the results of the current study are important toward understanding the mechanism of CDPK activation, many questions remain particularly in relation to the nature of the intramolecular binding of the CLD and JD. One important ambiguity lies in the role of the tether region consisting of the first 10 residues of the CLD (15). Mutations in this region have been
shown to affect intramolecular binding but remain insensitive to intermolecular interactions. Particularly intriguing is the question of possible concerted motions between the tether and linker regions. While our bimolecular approach has allowed for the study of changes between the steps of Ca$^{2+}$-binding and JD binding to the CLD, structural determination of a full-length JD-CLD construct is necessary to shed further insight into this region, although another study (32) with this aim has suffered from the same resonance broadening issues seen in this study. Another important question lies in the functional significance of the high degree of chemical exchange seen in the CLD. The structure elucidated from our studies may well be a transitory intermediate induced by the bimolecular interactions. Indeed bimolecular activation of truncated CDPK has been shown to be effective but still relatively poor (−34%) compared with the intact, full-length protein (10); however, it may be useful to separate the contributions derived from direct JD-CLD interactions and those outside of the binding pocket.

Taken as a whole our results intriguingly point to an unexpected mechanism by which the CLD and JD from CDPK interact. The non-CaM-like behavior of the protein, both conformationally and structurally, warrants mention in a more general context beyond the implications for calcium-regulatory proteins. As we move into an era of rapid structure determination and structure prediction ("structural genomics"), it is important to realize that sequence homology is a useful indicator only of secondary and rough tertiary structure. In this case, the >40% homology of the CLD to CaM is a metaphorical red herring in the understanding of how the protein behaves and functions. The degree of conformational exchange, the collapse of Ca$^{2+}$-CLD, and the 2–3 Å backbone r.m.s.d. of the well defined region demonstrate clear differences between CaM and the CLD and a closer functional resemblance to CNB. Especially if the long term goal of such projects is manipulation of fine structural details, such as rational drug design, rough tertiary structure is not adequate, and such proteins will need more than a cursory gene annotation based on homology to be useful.

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REFERENCES
1. Sanders, D., Pelloux, J., Brownlee, C., and Harper, J. F. (2002) Plant Cell 14, 415–420
2. Evans, N. H., McAinsh, M. R., and Hetherington, A. M. (2001) Curr. Opin. Plant Biol. 4, 387–392
3. Rudd, J. C., Legault, P., Vincent, S. J., Greenblatt, J., Konrat, R., and Kay, L. E. (1997) J. Am. Chem. Soc. 119, 6711–6712
4. Lee, J. Y., Yoo, B. C., and Harmon, A. C. (1998) Biochemistry 37, 6801–6809
5. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403–410
6. Bernstein, F. C., Koetzle, T. F., Williams, G. J., Meyer, E. F., Jr., Brice, M. D., Rodgers, R. J., Kennard, O., Shimanouchi, T., and Tasumi, M. (1977) J. Mol. Biol. 112, 535–542
7. Chou, J. J., Li, S., Kies, C. B., and Basu, A. (2001) Nat. Struct. Biol. 8, 990–997
8. Chattopadhyaya, R., Meador, W. E., Means, A. R., and Quiocio, F. A. (1992) J. Biol. Chem. 267, 1117–1129
9. Crivici, A., and Ikura, M. (1995) Annu. Rev. Biophys. Biomol. Struct. 24, 143–166
10. Gagne, S. M., Li, M. X., McKay, R. B., and Sykes, B. D. (1998) Biochem. Cell Biol. 76, 392–398
11. Meador, W. E., Means, A. R., and Quiocio, F. A. (1993) Science 262, 1718–1721
12. Griffith, J. P., Kim, J. L., Kim, E. E., Sinchak, M. D., Thomson, J. A., Fitzgbibbon, M. J., Fleming, M. A.,aron, P. R., Hsiao, K., and Navia, M. A. (1995) Cell 83, 507–522
13. Lee, S. Y., and Klevit, R. E. (2000) Biochem. Cell Biol. 78, 247–456
14. Yoshino, H., Izumi, Y., Sakai, K., Takezawa, H., Matsura, I., Maekawa, H., and Yazawa, M. (1996) Biochemistry 35, 2388–2393
15. Patsacolides, F. K., and Quiocho, F. A. (2000) Structure (Camb.) 11, 1303–1307
16. Basu, A., Gagne, S. M., Sykes, B. D., Ikura, M., and Clore, G. M. (1996) J. Magn. Reson. B 117, 1–2
17. Weljie, A. M., Yamazaki, T., Forman-Kay, J. D., and Kay, L. E. (1994) J. Magn. Reson. B 103, 197–201
18. Marston, S. M., Muhandiram, D. R., Yamazaki, T., Forman-Kay, J. D., and Kay, L. E. (1994) J. Am. Chem. Soc. 119, 7278–7287
19. Gagne, S. M., Li, M. X., McKay, R. B., and Sykes, B. D. (1998) Biochemistry 37, 6801–6809
20. Findlay, W. A., Martin, S. R., Beckingham, K., and Bayley, P. M. (1995) Biochemistry 34, 4295–4302
21. Yoo, B. C., Chen, X., Hsiao, K., and Navia, M. A. (1995) Nat. Struct. Biol. 12, 223–229
22. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) J. Biomol. NMR 6, 277–293
23. Johnson, B. A., and Blevins, R. A. (1994) J. Biomol. NMR 4, 603–614
24. Bax, A., Driscoll, P. C., Kay, L. E., Winglefield, P. T., Basu, A., Gagne, S., Clore, G. M., and Sykes, B. D. (1996) Biochemistry 35, 12222–12230
25. Zhang, X. S., and Choi, J. H. (2001) J. Mol. Biol. 53, 214–224
26. Harmon, A. C., Gribkov, M., Gubrium, E., and Harper, J. F. (2001) New Phytologist 151, 175–183
27. Weljie, A. M., Clarke, T. R., Uhrfjord, A. H., Harmon, A. C., and Vogel, H. J. (2000) Proteins 39, 343–357
28. Yoo, B. C., Lee, J. Y., and Harmon, A. C. (1998) Biochemistry 37, 7267–7277
29. Harmon, A. C., Yoo, B. C., and Lloyd, S. J. (1994) Biochemistry 33, 1514–1519
30. Shuker, S. B., Hajduk, P. J., Meadows, R. P., and Fesik, S. W. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 6467–6471
31. Lee, S. Y., Fitzgibbon, M. J., Fleming, M. A., Hsiao, K., and Navia, M. A. (1995) J. Mol. Biol. 252, 556–556
32. Roman, S. M., Muhandiram, D. R., Yasufuku, H., Hsu, C. B., and Basu, A. (1992) J. Mol. Biol. 236, 477–486