A Direct Test of the Reductionist Approach to Structural Studies of Calmodulin Activity

RELEVANCE OF PEPTIDE MODELS OF TARGET PROTEINS*

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James K. Kranz‡§, Eun K. Lee‡, Angus C. Nairn¶, and A. Joshua Wand‡

From the ‡The Johnson Research Foundation and Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6059 and the §Laboratory of Molecular and Cellular Neurosciences, The Rockefeller University, New York, New York 10021

Calmodulin is the prototypic transducer of the Ca\(^{2+}\) ion second messenger, present in all eukaryotes where it modulates numerous intracellular proteins, including a number of Ser/Thr protein kinases (1, 2). An elevation of intracellular Ca\(^{2+}\) concentrations results in saturation of calmodulin by calcium, inducing conformational changes in both N- and C-domains (3–6). In this activated state, Ca\(^{2+}\)-saturated calmodulin (CaM\(^3\)) binds to target enzymes, modulating their activity. The Ca\(^{2+}\)-dependent association is well studied, yet a mechanistic description of CaM function is confounded by the apparently diverse target sequences and numerous activities that are regulated by this protein (1, 7–9).

The structural paradigm of CaM signal transduction has been formed from a number of crystallographic and NMR studies, typically in the context of CaM binding to biochemically inactive peptide fragments from larger proteins (1, 9). This has been necessitated by the fact that the smallest intact enzymes that are regulated in CaM-dependent pathways are generally too large for standard NMR experiments and have been apparently resistant to crystallization efforts, forcing reliance on studies of the interaction of CaM with peptide fragments of calmodulin-binding domains. Indeed, peptide models are often used in many different types of studies of calmodulin-target protein interactions (2).

Commonly, CaM binds a single amphiphilic peptide, which forms an α-helix upon being sequestered from solvent via collapse of the two Ca\(^{2+}\)-binding domains of CaM around the peptide (10–15). Recently, structures of several large CaM-protein complexes have been determined (16–18), each suggesting that extrahelical interactions are available to CaM. In a study of a small conductance Ca\(^{2+}\)-activated K\(^+\) channel (SK2 channel) by Schumacher et al. (16), two molecules of CaM each span three helical segments in the tetrameric CaM-SK2 channel peptide complex. Similarly, Larsson et al. (17) found a multimeric complex between CaM and the transcription factor SEF2-1/E2-2. CaM has also been crystallized in association with a domain of Edema factor (EF) exotoxin from Bacillus anthracis (18). Remarkably, however, it is CaM that is engulfed by the EF domain in the ternary complex, with CaM bound as a new subdomain in the EF protein via “a molecular full nelson” (19). These examples indicate that CaM may be capable of numerous modes of interaction, further supporting a model of a conformationally adaptive protein. These studies suggest that CaM may utilize substantially larger surfaces in activating target enzymes than is indicated by CaM-peptide complexes. Thus a means for verifying the relevance of using a CaM-peptide mimetic in studies of the interaction between CaM and intact enzymes is needed.

Calmodulin-dependent protein kinase I (CaMKI) is part of a CaM-dependent kinase signaling cascade, comprised of CaMKK, CaMKI, and CaMKIV (20–22). A crystal structure of CaMKI(320) has provided insights into the structural basis for CaM-mediated activation of these and related enzymes (23).

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† To whom correspondence should be addressed: Dept. of Biochemistry & Biophysics, University of Pennsylvania, Philadelphia, PA 19104. Tel.: 215-573-7288; Fax: 215-573-7290; E-mail: wand@mail.med.upenn.edu.

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Many kinases are maintained in an autoinhibited state via interactions of the C-terminal regulatory domain with the catalytic core (24, 25). In CaMKI, the core catalytic domain (1–298) is flanked by a regulatory domain (299–320) that is comprised of overlapping pseudosubstrate and CaM-binding sequences. The regulatory domain inhibits enzyme activity through contacts of the pseudosubstrate sequence and the substrate-binding site and through contacts between the CaM-binding sequence and ATP-binding sites, thus providing an autoinhibitory mechanism (24, 25). The interaction between CaM and the CaM-binding domain sequesters the regulatory domain, releasing pseudosubstrate inhibition, and resulting in activation of the kinase.

In this work, we assay to what extent complexes of CaM with CaMKI(320) and with CaMKIp are similar at the level of the backbone structure of CaM. Studies comparing interactions of CaM with intact CaMKI versus a synthetic CaMKIp peptide (residues 299–320) show largely similar spectroscopic changes, and a similar responsiveness to mutations (26, 27), providing support for the use of peptides as models of CaM interactions. Here, the extraordinary sensitivity of the NMR chemical shift is used to report on the subtle structural changes that occur throughout the entire calmodulin molecule upon interaction with a target peptide or protein. The observed chemical shifts show that CaM-CaMKIp and CaM-CaMKI(320) are virtually identical in terms of backbone structure.

**EXPERIMENTAL PROCEDURES**

**Protein Purification and Sample Preparation—** Recombinant chicken CaM was prepared as described previously (28). For overexpression of $^{13}$C,$^{15}$N-labeled CaM, cells were grown on minimal medium containing 0.1% $^{2}$H$_2$O with 0.2% (U-$^{13}$C$_6$-$^{15}$N$_2$) glucose (Isotec). For overexpression of [H, $^{13}$C,$^{15}$N]CaM, cells were first adapted to growth in minimal media containing 50% $^{2}$H$_2$O, then 99% $^{2}$H$_2$O over successive 24-h periods, followed by overexpression in minimal medium containing 0.1% $^{15}$NH$_4$Cl and 99% $^{2}$H$_2$O (Isotec). Following purification, the amide protons were equilibrated to 99% $^{2}$H$_2$O by incubating [H, $^{15}$N]CaM at 55 °C for ≈48 h in NMR buffer (below).

The DNA encoding CaMKI(320) was subcloned from a previously described fusion protein expression system (23). The CaMKI(320) DNA was inserted after a hexahistidine N-terminal leader coding sequence in the pET-15b vector (Novagen) utilizing NdeI and BamHI restriction sites, using standard methods. Overexpression of recombinant CaMKI(320) in Escherichia coli BL21(DE3) cells was similar to that described previously (23), with all growth performed on minimal medium and 1 mM isopropyl-$β$-D-thiogalactopyranoside induction, cells were harvested, lysed by sonication, and clarified by centrifugation. The His-tagged kinase was isolated on a nickel-nitrilotriacetic acid His-bind resin, washed with wash buffer, followed by 1:1000 saturation of NiSO$_4$ in D$_2$O. $^{13}$C and $^{15}$N chemical shifts were determined via the TROSY spectrum has been offset by $^{15}$NH to compensate for the selection of single component of the NH cross-peak quartet. 

**RESULTS AND DISCUSSION**

**CaM Binding to CaMKI Enzyme and Peptide—** Previous studies have shown the interaction of CaM with CaMKI protein and peptides requires Ca$^{2+}$, involves sequestration of Trp$^{303}$ from solvent, and binding of CaM to a helical peptide (23, 24, 26, 27). Backbone resonance assignments of Ca$^{2+}$-saturated $^{13}$C,$^{15}$N-labeled CaM bound to CaMKI(320) complex (BMRB number 5287) were determined by reference to the CaM-CaMKIp complex. Backbone assignments for CaM(39) and CaM-smMLCKp complex (40, 41) are known. All spectra were processed using FELIX (Molecular Simulations Inc.).
chemical shift differences between CaM-CaMKIp and free CaM are suggestive of substantial structural changes upon peptide association (Fig. 3A), consistent with chemical shift changes and structural rearrangements observed in formation of other CaM-peptide complexes (10–15, 42).

Studies of “intact” CaMKI have utilized a truncated version of the enzyme (1–320) that results from proteolytic cleavage during expression and purification of the full-length enzyme (23). This truncated form is fully autoinhibited, includes the complete consensus CaM-binding sequence (9), displays CaM-dependent activation (24), and is the same form of the protein as was utilized in crystallization studies (23). A standard 15N HSQC spectrum of the CaM-CaMKI(320) complex showed significant line-broadening of CaM resonances (data not shown), requiring the use of NMR strategies suitable for studies of large (>35 kDa) proteins. The TROSY-HSQC (38) spectrum of [13, 42].

CaM(1–320) is virtually identical to that of the CaM-CaMKI(320) complex (Fig. 1), allowing complete cross-assignment of the CaM-CaMKI(320) spectrum by reference to the assigned spectrum of the CaM-CaMKI complex. The chemical shift differences between these two are on the order of the precision of individual chemical shifts, clearly denoting that CaM adopts virtually identical structures in association with either the CaMKI enzyme or peptide (Fig. 3D). Substantial line-broadening is observed in HSQC spectra of the CaM-CaMKI(320) complex, which indicates the hydrodynamic properties of CaM are influenced by association with the comparatively large CaMKI enzyme. However, from the perspective of backbone chemical shifts, there is no difference in the backbone structure of CaM when bound to the 35-kDa enzyme or when attached to the 22-amino acid peptide model.

Comparison of CaM-CaMKI with CaM-smMLCKp—How unique is CaMKI sequence in promoting changes in the structure of CaM? Is the similarity between CaM-CaMKIp and CaM-CaMKI(320) expected for all CaM-enzyme complexes? To address these issues, we compared the effect of 1H and 15N chemical shift changes in CaM upon binding two different recognition sequences, CaMKIp and a distantly homologous CaM-binding peptide from smooth muscle myosin light chain kinase (smMLCKp). Both interactions are known to require Ca2+ and are known to interact with similar sites on CaM (12, 26, 27). A comparison of CaM-CaMKIp relative to free CaM (Fig. 3A) with chemical shift changes in CaM-smMLCKp relative to free CaM (Fig. 3B) indicates that binding to either peptide produces effects of significant magnitude throughout CaM but with a quite different distribution. A comparison of the crystal structures of free CaM and CaM-smMLCKp shows the change in backbone conformation is small within either N-terminal (0.43Å r.m.s. deviation) or C-terminal domain (0.55Å r.m.s. deviation).2 Despite small changes in structure, substantial chemical shift changes are induced upon peptide binding; CaM chemical shifts are extremely sensitive to the small conformational changes that take place upon complexation. A comparison of amide chemical shifts from CaM-CaMKIp and CaM-smMLCKp shows substantial effects over the entire sequence of CaM (Fig. 3C), with larger chemical shift differences for residues in the central helix, indicating that CaM can differentiate between the substrates. In this context, the lack of differences in the chemical shifts of CaM in the CaM-CaMKIp and CaM-CaMKI(320) enzyme complexes are even more striking.

In light of the intrinsic sensitivity of backbone chemical shifts to perturbations in the structure of CaM, the equivalence of 1H and 15N chemical shifts of CaM-CaMKIp and those of CaM-CaMKI(320) indicates no differences in the structure of CaM (Fig. 3D) bound to these two substrates. While our results do not presently address changes induced in the structure of the intact CaMKI enzyme upon CaM association, the data are consistent with a model whereby interactions between CaM and CaMKI are limited to the CaM recognition sequence in the regulatory domain. While this is likely also true for some CaM-regulated proteins, there is evidence from other systems that the mechanism of CaM activation can be more complex than could be extrapolated from peptide binding studies (18, 43, 44).
Validity of Peptide Models of Calmodulin-binding Domains

However, comparisons of binding energies of CaM to either peptides or enzymes may not take into account possible interactions between the regulatory domain and the catalytic domain that must be disrupted upon CaM binding. Here we have demonstrated the feasibility of directly examining this issue in large protein complexes using TROSY-based NMR methods. Furthermore, the work presented here strengthens the model for CaM-dependent activation of an autoinhibited CaMKI enzyme, relieving allosteric inhibition by sequestering the regulatory domain, preventing its interaction with both the substrate and ATP binding domains. In this activated state, CaM is a silent partner unaware of the presence of an active enzyme tethered to the CaM recognition sequence.

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