THE DISORDERED REGULATION OF CALCINEURIN: HOW CALMODULIN-INDUCED REGULATORY DOMAIN STRUCTURAL CHANGES LEAD TO THE ACTIVATION OF CALCINEURIN

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THE DISORDERED REGULATION OF CALCINEURIN: HOW CALMODULIN-INDUCED REGULATORY DOMAIN STRUCTURAL CHANGES LEAD TO THE ACTIVATION OF CALCINEURIN

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
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Lexington, Kentucky

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ABSTRACT OF DISSERTATION

THE DISORDERED REGULATION OF CALCINEURIN: HOW CALMODULIN-INDUCED REGULATORY DOMAIN STRUCTURAL CHANGES LEAD TO THE ACTIVATION OF CALCINEURIN

Calcineurin (CaN) is a highly regulated Ser/Thr protein phosphatase that plays critical roles in learning and memory, cardiac development and function, and immune system activation. Alterations in CaN regulation contribute to multiple disease states such as Down syndrome, cardiac hypertrophy, Alzheimer’s disease, and autoimmune disease. In addition, CaN is the target of the immunosuppressant drugs FK506 and cyclosporin A. Despite its importance, CaN regulation is not well understood on a molecular level. Full CaN activation requires binding of calcium-loaded calmodulin (CaM), however little is known about how CaM binding releases CaN's autoinhibitory domain from the active site. Previous work has demonstrated that the regulatory domain of CaN (RD) is disordered. The binding of CaM to CaN results in RD folding. Folding of the RD in turn causes the autoinhibitory domain (AID) located C-terminal to the RD to be ejected from CaN’s active site. This binding-induced disorder-to-order transition is responsible for the activation of CaN by CaM. In this work, we explore the nature of the disorder in the RD and its transition to an ordered state, demonstrating that the RD exists in a compact disordered state that undergoes further compaction upon CaM binding. We also demonstrate that a single CaM molecule is responsible for binding to and activating CaN. Finally, we determine that the CaM binding to CaN induces an amphipathic helix (the distal helix) C-terminal to the CaM binding region. The distal helix undergoes a hairpin-like chain reversal in order to interact with the surface of CaM, resulting in the removal of the AID from CaN's active site. We employ site-directed mutagenesis, size-exclusion chromatography, protein crystallography, circular dichroism spectroscopy, fluorescence anisotropy and correlation spectroscopy, and phosphatase activity assays to investigate the ordering of CaN’s regulatory domain, the stoichiometry of CaN:CaM binding, and the impact of the distal helix on CaM activation of CaN.
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Chapter 1: Background and Introduction

Calcineurin: an overview

Calcineurin (CaN) is a heterodimeric calcium/calmodulin activated serine/threonine protein phosphatase. It was originally identified by three separate groups: Wang and Desai,\textsuperscript{1} Watterson and Vanaman,\textsuperscript{2} and Klee and Krinks.\textsuperscript{3} Each group identified CaN as an inhibitor of the calmodulin (CaM) activated cyclic 3’, 5’-nucleotide phosphodiesterase. The reason for CaN’s apparent inhibition of the cyclic 3’, 5’-nucleotide phosphodiesterase is that CaN binds to and thereby depletes CaM, preventing activation of cyclic 3’, 5’-nucleotide phosphodiesterase by CaM.

It was later learned that CaN is a calmodulin activated protein phosphatase and has a multitude of targets and interacting proteins. CaN is well conserved among eukaryotes. In lower eukaryotes, such as \textit{S. cerevisiae}, CaN functions in pathways for growth, motility, stress response, and calcium homeostasis, among others.\textsuperscript{4} In humans CaN has many targets across most tissue types, but CaN’s most well-known target is the nuclear factor of activated T-cells (NFAT) family of transcription factors. It is CaN’s activation of NFATs that makes CaN the target of the immunosuppressant drugs that are given to patients who have had organ or tissue transplants to avoid rejection of the transplant.\textsuperscript{4,5}

Besides immune system involvement, CaN plays a role in cardiac, vasculature, nervous system, and musculature development. CaN signaling is also necessary for learning and memory, skeletal muscle growth, and immune system activation.\textsuperscript{4} Because of its roles in these varied signaling systems inappropriate CaN regulation has been implicated in a number of pathological states including Alzheimer’s disease,\textsuperscript{6} Down syndrome,\textsuperscript{7} and cardiac hypertrophy.\textsuperscript{8,9}
Calcineurin Signaling

**Nuclear factor of activated T-cells (NFAT)**

The nuclear factor of activated T-cells (NFAT) family of transcription factors is composed of five members, NFAT1-5 (or NFATc1-c5), and CaN regulates NFATs 1-4. The NFATs are transcription factors that can activate a variety of gene programs depending on the tissue type, NFAT type, number and location of NFAT phosphorylations, and binding partner that the NFAT associates with when in the nucleus. Under resting cell conditions NFAT is maintained inactive in the cytosol by multiple phosphorylations that prevent use of the NFAT nuclear localization signal (NLS). The number and location of the phosphorylations also determine NFAT’s affinity for DNA, but the mechanism by which phosphorylations determine DNA affinity and hide the NLS are unknown.

The NFATs interact with CaN via a conserved ~300 amino acid sequence that is encoded by a single exon. This CaN interacting domain begins ~100 residues from the N-terminus and is N-terminal to the DNA binding region (Figure 1.1). The CaN interacting domain includes two serine rich gatekeeper regions (SRRs) and three SPxx motifs, all of which can have multiple phosphorylations. Additionally, this region contains two CaN interacting motifs: a PxIxIT motif and a LxVP motif. The PxIxIT forms a β-strand that lines up with and completes a β-sheet on the outer edge of CaN’s catalytic domain. The LxVP motif interacts with CaN at the interface of the two CaN subunits. These two docking sites are on the same face of CaN as the active site but opposite sides of the catalytic domain. NFAT’s phosphorylations are in the region between these two motifs, thus the CaN binding motifs act as anchors to bring the phosphorylation sites in proximity to CaN’s active site (Figure 1.2). Many other CaN targets employ one or both of these binding motifs to dock with CaN. The affinity of substrates for CaN seems to be determined by the variable amino acids in the PxIxIT or LxVP motif that the substrate uses. It is thought that these docking sites are what determine CaN’s substrate specificity.
When intracellular calcium levels rise, CaN is activated by calcium/calmodulin and can then dephosphorylate NFAT. CaN dephosphorylation of sites in NFAT’s CaN interacting domain exposes the NFAT NLS which is located in the same domain. Once NFAT’s NLS is revealed it is relocated from the cytoplasm to the nucleus.¹⁴⁻¹⁸ In the nucleus NFATs can bind DNA in monomer form, as a homodimer, or as a heterodimer with a number of different transcription factor partners: AP-1 (Fos-Jun heterodimer), GATA4, and MEF2 to name a few.⁵,²³⁻²⁵ The dimer partner helps to determine which genes are affected by NFAT and allows for integration of multiple signaling pathways.

Once in the nucleus and partnered with the appropriate partner, NFAT acts as a transcription factor or repressor to up-regulate or down-regulate entire gene programs. NFAT acts on distal promotors, and its consensus sequence is (A/T)GGAAA.⁵ When NFAT is paired with other transcription factors, the dimer binds to composite sites with both the NFAT consensus sequence and the sequence for the NFAT partner.¹¹ The various NFATs often have overlapping functions, and deletion of two or more NFAT types are usually necessary to observe an altered phenotype.²⁶

Calcineurin/NFAT signaling is necessary for proper fetal development and adult homeostasis in many calcium sensitive tissues including the heart, vasculature, nervous system, immune system, skeletal muscles, bone, pancreatic β-cells, and kidneys.¹³ During fetal heart development, CaN dephosphorylation of NFAT3 and NFAT4 leads to an increase in cardiac contractile proteins and oxidative metabolic proteins; signaling through NFAT1 is necessary for valve development; and signaling through NFAT2 is involved in angiogenic remodeling.⁹,²⁴,²⁷ CaN/NFAT signaling was discovered via its role in the immune system and is very well studied in this system.²⁸,²⁹ NFATs are necessary for differentiation of T-cells, and NFAT1 and NFAT2 are required for cytokine production in stimulated T-cells.³⁰ In skeletal muscles, CaN/NFAT signaling, under conditions of chronic muscle activity, induces expression of myosin heavy chain 1 (MyHC1). MyHC1 expression is necessary for muscle fiber switching from fast-twitch to slow-twitch fibers.¹¹ NFAT2 activity is essential for osteoclast progenitor differentiation in bones.³¹,³²
pancreatic β-cells the insulin gene has a known NFAT promotor, and β-cell specific depletion of CaN in mice results in age-dependent diabetes.\textsuperscript{33-35} CaN signaling through NFAT is necessary for renal development at the earliest stages and plays a role in salt and water homeostasis.\textsuperscript{36-38}

The above examples are just a fraction of the signaling pathways under the influence of CaN/NFAT signaling. Additionally, CaN has a multitude of targets besides NFAT in these and other systems. In the following section I will discuss CaN’s well known, if not fully understood, role in long-term depression at excitatory synapses. This example is given in order to explore important non-NFAT CaN signaling.

\textit{Long-Term Depression at Excitatory Synapses}

Long-term potentiation (LTP) and long-term depression (LTD) are mechanisms used at the post-synaptic neurons of excitatory synapses to sensitize or desensitize, respectively, the receiving neuron to excitation stimuli. LTP strengthens the connection between neurons while LTD weakens it. These processes are important in learning and memory. LTP and LTD are very complex and the mechanisms for LTP and LTD induction can be different depending on the type and architecture of the synapse. What follows is a brief overview of the role CaN plays in LTD.\textsuperscript{39}

The NMDA receptor (NMDAR), calcium-permeable AMPA receptor (AMPAR), and calmodulin activated kinase II (CaMKII) are important in LTP.\textsuperscript{39-43} NMDAR and AMPAR are both glutamate receptor cation channels located on post-synaptic neurons. NMDAR is also a voltage-gated channel. When glutamate binds the receptors AMPARs are opened, allowing the influx of cations, including calcium. In addition to glutamate binding, NMDAR requires depolarization of the post-synaptic neuron to open. Depolarization releases a magnesium ion in NMDAR pores, allowing the influx of cations.\textsuperscript{39-41} LTP can occur if the excitatory stimuli is strong enough causing both receptors to open, and the influx of calcium through NMDARs allows the activation of CaMKII. CaMKII then phosphorylates AMPARs, making them more sensitive to excitatory stimuli, thereby
reinforcing the stimulus. CaMKII also assists in increasing the number of AMPARs at the membrane.\textsuperscript{39,41,42}

LTD occurs when a persistent, weak excitatory stimulus is applied to the post-synaptic neuron. This opens AMPARs, allowing a cation influx that is not strong enough to depolarize the cell. Lack of depolarization maintains NMDARs in their closed state so that CaMKII is inactive.\textsuperscript{39} CaN is anchored to the dendritic membrane by the scaffolding protein AKAP79.\textsuperscript{12} When AMPARs allow the influx of calcium, CaN is activated and dephosphorylates AMPARs, desensitizing them to excitatory stimuli.\textsuperscript{12,43} CaN activity may supersede CaMKII activity under these circumstances because, while both are calcium/calmodulin activated, CaN has a much higher affinity for CaM than CaMKII does (K\textsubscript{D} \textasciitilde 1pM and \textasciitilde 300nM, respectively) and because CaMKII is associated with NMDARs when inactive, not AMPARs.\textsuperscript{44,45} While, as mentioned, LTP and LTD are much more complex than the brief explanation given, CaN’s role in LTD is its dephosphorylation of AMPARs, and this dephosphorylation is crucial for LTD.

\textit{CaN Signaling in Human Health}

We have discussed CaN’s role in the development and maintenance of several human organ/tissue systems, so we can see how altered CaN signaling can lead to a host of different pathological states. CaN mis-regulation has been implicated in cardiac hypertrophy,\textsuperscript{8,9} Alzheimer’s disease,\textsuperscript{6} and Down syndrome.\textsuperscript{7}

When CaN/NFAT signaling is chronically activated in adults, the fetal gene program is reactivated, and it leads to cardiac hypertrophy which is a leading predictor of congestive heart failure. In fact, constitutively activated CaN, NFAT3, or NFAT4 can induce massive cardiac hypertrophy in mice, including increased myocyte size, an increase in collagen and fibrous tissue, and often congestive heart failure or sudden death.\textsuperscript{9,24,27}

CaN has been shown to dephosphorylate Tau protein. The hyperphosphorylated form of Tau can form neurofibrillary tangles, a hallmark of Alzheimer’s disease in human brain. CaN activity is shown to be decreased in the brains of Alzheimer’s disease patients.
Decreased CaN activity could lead to hyperphosphorylated Tau, and, thereby, neurofibrillary tangles.\textsuperscript{6,46}

Regulator of calcineurin 1 (RCAN1) is an endogenous regulator of CaN.\textsuperscript{47} The gene for RCAN1 is found in the Down syndrome critical region (DSCR) of chromosome 21, and is overexpressed in individuals with Down syndrome.\textsuperscript{48} Overexpressed RCAN1 chronically inhibits CaN. Mental retardation, muscle hypotonia, and immune deficiency are all characteristic Down syndrome pathologies.\textsuperscript{49} It is thought that chronic CaN inhibition by RCAN1 contributes to these pathologies.\textsuperscript{7,48} As mentioned in the previous CaN signaling sections, CaN contributes to learning and memory, skeletal muscle development and maintenance, and immune system activation. Additionally, Down syndrome patients almost invariably develop early-onset Alzheimer’s disease if the patient lives past \textasciitilde35 years.\textsuperscript{7,49} It is possible that decreased CaN activity due to increased RCAN1 levels contributes to this occurrence of Alzheimer’s disease.

Finally, CaN is the target of the immunosuppressant drugs FK506 (also known as tacrolimus) and cyclosporin A.\textsuperscript{18,50} These drugs are necessary for patients who have had tissue or organ transplants as they help to prevent rejection of the transplant. In cells, FK506 binds FKBP12 (FK506 binding protein 12), and cyclosporin A binds cyclophilin. These proteins are prolyl-isomerases. The binding of the drugs to their protein partners allows the complex to then bind CaN, preventing NFAT binding and dephosphorylation.\textsuperscript{51-54} Without NFAT signaling, T-cells are not activated, which allays immune system attack on transplanted tissues.

The vast network of CaN/NFAT signaling, CaN signaling in the very important process of LTD, and the impact of altered CaN signaling on human health demonstrates the large scope and importance of CaN function. However, despite extensive studies concerning CaN there is much we do not understand about the protein itself and its activation by calcium-loaded calmodulin (CaM). In the following sections, we will explore what is known about CaN structure and activation as well as some of the aspects of CaN structure and activation that we do not fully understand.
Calcineurin Structure and Activity

CaN is a heterodimer composed of a 57-61kDa A chain (CnA) and a 19kDa B chain (CnB). CnA is composed of the catalytic domain, CnB binding helix (CnBBH), regulatory domain (RD), autoinhibitory domain (AID), and C-terminal tail (CT) (Figure 1.3). At low calcium concentrations the AID resides in the catalytic site of CnA, keeping the enzyme inactive. When calcium levels rise, calcium loaded CaM binds to the CaM binding region (CaMBR) located in the regulatory domain of CnA. CaM binding releases the AID from the catalytic site, activating CaN (Figure 1.4). CnB is a calcium binding EF hand protein that is structurally homologous to CaM. While CnB can bind up to four calcium ions, it is constitutively bound to CnA, regardless of calcium concentrations. There are three CaN isoforms: αCaN is found primarily in neurons, βCaN is ubiquitous, and γCaN is testes specific. The differences in regulation and activity between isoforms is unclear. In this work CaN refers to αCaN.

Calcineurin A

The catalytic domain of CnA has a β-sandwich fold consisting of two central β-sheets surrounded by a mixture of α-helices and β-sheets (Figure 1.3). This architecture is consistent with the phosphoesterase motif common in enzymes involved in hydrolyzing phosphoester bonds including protein phosphatase 1, protein phosphatase 2a, alkaline phosphatase, and phosphodiesterase. The active site of CaN has a dinuclear metal center consisting of Fe$^{3+}$-Zn$^{2+}$. The Fe$^{3+}$ is coordinated by Asp90, His92, Asp118, and three waters. The Zn$^{2+}$ is coordinated by Asn150, His199, His 281, Asp118, and a water that it shares with the Fe$^{3+}$. In addition, it is thought that Arg122 and Arg254 are necessary to help stabilize the negative charge from phosphate in the active site during catalysis. It is believed that the catalysis proceeds via direct transfer of the phosphate moiety to a metal activated water molecule. The metal activated water initiates a nucleophilic attack on the phosphate group, assisted by the two histidines.

Beyond the catalytic domain, which is the N-terminal portion of CnA, there is the CnBBH where CnB binds. C-terminal to the CnBBH is the regulatory domain (RD). The
RD is a disordered stretch of CnA which houses the CaMBR and will be discussed in more detail below. At the end of the RD is the AID which exists as a distorted helix in the catalytic site in the CaN crystal structure. Beyond the AID is the C-terminal tail (CT) which is disordered and has no known function (Figure 1.3).

**Calcineurin B**

CnB is a calcium binding EF hand protein that is homologous to CaM. It has an N-terminal lobe and a C-terminal lobe, each of which binds two calcium ions. The C-terminal lobe binds calcium with high affinity and is considered to be constitutively calcium and calcineurin bound. The N-terminal lobe has a lower calcium affinity and must bind calcium for CaN to have efficient phosphatase activity against peptide substrates. CnB binds the CnBBH of CnA in an antiparallel fashion. The C-terminal lobe binds to the N-terminal portion of the CnBBH and is adjacent to the CnA catalytic domain while the N-terminal lobe binds to the C-terminal portion of the CnBBH.

Studies suggest that CnB plays a regulatory role in CaN function. Without calcium (in the presence of EGTA) limited proteolysis with chymotrypsin cleaves the CnB linker and first helix, but the CaMBR containing RD, is protected. Conversely, in the presence of calcium, all of CnB is protected and the RD is rapidly degraded. This suggests that a portion of the RD interacts with the CnBBH when the N-terminal lobe of CnB is not calcium bound, but a conformational change occurs upon calcium binding that releases the RD, likely so that it is available for interaction with CaM.

CnB also appears to impact substrate LxVP motif interaction with CaN’s LxVP docking site which is critical for substrate dephosphorylation and thought to be a mode of substrate recognition. The LxVP docking site is found at the CnA-CnB interface. Substrate only binds the LxVP site of active CaN. A truncated CnA (catalytic domain and CnBBH only) with CnB bound shows normal $V_{\text{max}}$ levels with a peptide substrate, as would be expected without the AID in the active site. However, the $K_{\text{m}}$ of this truncated CaN is five times higher than active, native CaN. Addition of calcium (binding the N-terminal lobe of CnB) reduces the $K_{\text{m}}$ to native levels. This suggests that CnB calcium
binding affects CaN substrate affinity, giving CnB a regulatory, not just structural, role in the CaN heterodimer.

**Calcineurin Activation**

Under resting calcium conditions, CaN is maintained in an inactive state by its autoinhibitory domain (AID) bound to CaN’s catalytic site. When calcium levels rise, calmodulin (CaM) binds calcium and then binds to the calmodulin binding region (CaMBR) of CaN, located in the regulatory domain (RD) (Figure 1.4).\(^{56}\)

CaM is a 148 amino acid, 16.7kDa protein that is a major transducer of calcium signaling in cells.\(^{65}\) CaM binds to and regulates ~300 cellular targets.\(^{66}\) CaM is composed of two lobes connected by a flexible linker, often referred to as a dumbbell shape (Figure 1.5a). CaM has four calcium binding sites, two in each lobe. While there are several types of sequences that CaM binds to, calcium loaded CaM preferentially binds to basic, amphipathic, \(\alpha\)-helical sequences (BAA sequences).\(^{44,66,67}\) The CaMBR of CaN is a BAA sequence.\(^{66,68}\) In some cases, as with CaN, the CaM target sequence is disordered but prone to \(\alpha\)-helicity, as measured by the ability of 2,2,2-Trifluorethanol (TFE) to induce helix in the sequence.\(^{69}\) TFE is well known to induce \(\alpha\)-helical structure in peptides possessing a propensity to be helical.

Typically, when CaM binds BAA sequences, the CaM central linker helix becomes flexible and its two CaM lobes wrap around the target to form a compact ellipsoid (Figure 1.5b).\(^{66,67}\) The manner in which CaM binds CaN is still not completely understood. Crystal structures of CaM bound to a peptide of the CaMBR of CaN done by Ye et. al. and Majava et. al show CaM bound to CaN in a 2:2 manner with one lobe of CaM bound to one end of a CaMBR peptide while the other lobe is bound to another CaMBR (Figure 1.6). However, small angle x-ray scatter (SAXS) and size exclusion chromatography (SEC) performed by Majava et al. demonstrated a 1:1 CaM:CaMBR complex.\(^{70}\) Native-gel analysis and SEC on CaM:CaMBR by Ye et al. showed both a 2:2 complex and a 1:1 complex, with 1:1 being the dominate species. Also, through extensive hydrodynamic studies, O’Donnell et al. demonstrate a 1:1 CaM:CaMBR complex that
likely exhibits the canonical wrap-around CaM binding.\textsuperscript{44} This leaves some uncertainty about the physiological stoichiometry of CaM binding to CaN, at important aspect of CaM regulation of CaN for us to understand.

CaN’s CaMBR is located in the regulatory domain (RD) of CaN. The CaMBR begins 20 residues from the end of the CnBBH and ends 52 residues from the beginning of the AID. Trypsin digests and other hydrolysis experiments by Manalan and Klee, and Yang and Klee, respectively, demonstrated that the RD is readily hydrolyzed, showing that it is flexible and accessible in nature.\textsuperscript{58,71} In the crystal structure of full length CaN the electron density for the RD and C-terminal tail (CT) are missing suggesting that these regions are highly mobile.\textsuperscript{52} Additionally, Romero and Dunker noted that the sequence of the RD, enriched in charged and polar residues, gives it the potential to be disordered.\textsuperscript{72}

Work by Rumi-Masante et al. showed by H/D exchange mass spectrometry and circular dichroism spectroscopy that a fragment corresponding to CaN’s RD, AID, and CT (RD-AID-CT) is disordered in the absence of CaM, though the AID is well-ordered in full-length, inactive CaN.\textsuperscript{73} The RD portion of this fragment contains the CaM binding region and the 52 residues that separate it from the AID (Figure 1.7). Canonically, CaM’s two lobes wrap around its target site and induce $\alpha$-helix in that sequence. Knowing now that the overall RD is disordered, this leaves 52 residues of disorder between the end of the CaMBR and the beginning of the AID, suggesting that CaM binding would not be able to forcibly remove the AID from the active site. How then does CaM activate CaN with its CaMBR and AID 52 residues apart and with those 52 residues being disordered?

The trypsin digests performed by Manalan and Klee, aside from demonstrating that the RD is flexible and accessible to proteases, also suggested that the RD of CaN undergoes a large conformational change upon CaM binding as the highly unprotected RD becomes protected from trypsin digest when CaN is preincubated with CaM.\textsuperscript{71} Rumi-Masante et al. also showed that upon CaM binding, ~50 residues within the RD become protected from H/D exchange and that the RD gains approximately fifty residues of $\alpha$-helicity.\textsuperscript{73} About half of these residues reside in the CaMBR while the remainder are C-terminal to the
CaMBR, between it and the AID. The high protection and α-helicity of the CaMBR when CaM is bound is expected given the very tight binding between CaM and CaN (KD ~1pM) and the manner in which CaM typically binds its targets. The protection and helicity in the region between the CaMBR and AID, suggested by RD protection in Manalan and Klee’s CaN/CaM trypsin digests and confirmed by Rumi-Masante et al., suggests a larger scale structuring than just the sequence CaM directly binds.

Exploration of the Regulatory Domain and Regulatory Domain/CaM interactions: Dissertation Overview

CaN signaling has been demonstrated to be important in many signaling pathways and tissues. Its interactions have been well studied, especially given its importance as the target for immunosuppressant drugs. However, there is still much we do not understand about the CaN protein itself, specifically concerning CaN’s regulatory domain (RD) interaction with CaM. Through work from multiple groups, we know that the RD is disordered in the absence of CaM but undergoes an ordering conformational change upon CaM binding. Beyond this, there is much we do not understand about the interaction.

What is the shape of the RD alone? To term a protein or a protein region “disordered” only tells us that the protein does not adopt a well-ordered tertiary structure. Within this definition, the protein can exist anywhere from an ensemble of completely disordered string-like shapes to a molten-globule shape that has secondary structure, but where the tertiary structure is ill-defined. We know from the work done by Rumi-Masante, et al. that the RD is not on the molten-globule end of the disorder spectrum as CD spectra of the RD -AID-CT indicate it has no persistent secondary structure. However, the question remains, is the RD more compact in shape or extended? Chapter three of this work explores the shape of the RD in the absence of CaM in order to help characterize its disordered state. Chapter three also investigates the overall shape of the CaM:RD complex. Fluorescence anisotropy, fluorescence correlation spectroscopy (FCS), and circular dichroism (CD) spectroscopy are all employed in this endeavor.
Chapter four delves into the stoichiometry of the CaM:RD complex. As noted, the stoichiometry of this complex seems to exist at 2:2 in crystal structures; at both 2:2 and 1:1 in solution work, with 1:1 being the dominant species; and at 1:1 in hydrodynamic studies.\textsuperscript{44,70,74,75} In an attempt to reconcile these views I employ crystallography to investigate the structure of CaM bound to a peptide of CaN’s CaMBR (pCaN), and size exclusion chromatography (SEC) is used to explore the CaM:RD stoichiometry. RD is used in the SEC to determine if using a larger portion of CaN than just the CaMBR would impact the stoichiometry of CaM binding.

Rumi-Masante, et al. demonstrated that the RD-AID-CT undergoes an ordering upon CaM binding that extends beyond the CaMBR.\textsuperscript{73} There are \textasciitilde50 residues that gain structure upon CaM binding while the CaMBR is 24 residues in length. The remaining order is gained in the region between the end of the CaMBR and the beginning of the AID and is $\alpha$-helical.\textsuperscript{73} Chapter five of this work investigates the nature of this ordering and its impact on CaN activity using site-directed mutagenesis, CD spectroscopy, CD thermal melts, and CaN phosphatase activity assays.

Chapter six serves as summation and further interpretation of the data presented in previous chapters. Future experiments are also suggested to expand upon this work and to further clarify the CaM:RD interaction both on its own and in the full context of calcineurin.

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Figure 1.1 NFAT domains and phosphorylations

Figure 1.2 Schematic of NFAT docked to CaN

Figure 1.3 Calcineurin domain structure

Structure of CaN with domains highlighted. PDBID: 1AUI.\textsuperscript{52} Catalytic domain: dark blue; CnB binding helix: blue; Regulatory domain (RD): green, drawn, electron density is missing from crystal structure; CaM binding region: orange rectangle, drawn, electron density is missing from crystal structure; Autoinhibitory domain (AID): red; C-terminal tail (CT): light gray, drawn, electron density is missing from crystal structure; Calcineurin B (CnB): dark gray.
Figure 1.5 CaM structure

Calmodulin: green, MLCK CaM binding peptide: blue, calcium: red. a) Crystal structure of CaM in the classical calcium loaded dumbbell form. PDB ID: 3CLN.\textsuperscript{76} b) NMR structure of CaM bound to a peptide of a canonical CaM binding site in MLCK. PDB ID: 1CDL.\textsuperscript{77}
Figure 1.6 Structure of CaM with CaMBR of CaN, 2:2 stoichiometry

Crystal structure showing two CaM molecules bound to two peptides of the CaMBR from CaN. One CaM molecule is shown in orange, and the other is shown in yellow. CaMBR peptides from CaN are shown in red. PDB ID: 2W73.70 Adapted and reprinted with permission from PLOS: Majava, V., & Kursula, P. (2009). Domain Swapping and Different Oligomeric States for the Complex Between Calmodulin and the Calmodulin-Binding Domain of Calcineurin A. (V. Majava & P. Kursula, Eds.) PLoS ONE, 4(4), e5402. doi:10.1371/journal.pone.0005402.t003 License: Creative Commons
Figure 1.7 Schematic of CaN domains and CaM binding region placement within the regulatory domain

Chapter 2: Materials and Methods

Materials

All chemicals were obtained from Sigma (St. Louis, MO) and were of the highest purity, except where noted.

Plasmid expression vectors

The plasmid pETagHisCN containing the human αCaN A (with N-terminal His6 tag) and B1 genes, together forming αCaN, was obtained from Addgene (Cambridge, MA). The pETCaMI expression vector was generously provided by Professor Anthony Persechini of the University of Missouri at Kansas City. An E. coli codon-optimized gene for the human sequence RD construct was synthesized by Genscript (Piscataway, NJ). The RD gene has added bases coding for MWG at the N-terminus and GCGGGLE at the C-terminus (Figure 2.1). The extra MWG creates the methionine start codon and allows for protein concentration determination via the added tryptophan. The GCGGGLE creates a glycine linker to the His6 tag in the expression plasmid, adds a cysteine for the attachment of maleimide fluorophores, and adds an XhoI restriction site. The RD was subcloned into the pET303/CT-His vector that adds a C-terminal His6 tag (Invitrogen, Carlsbad, CA) by using XbaI and XhoI to excise the RD gene from its original plasmid and to linearize the pET303/CT-His vector and ligating the RD gene to linearized pET303/CT-His. The subcloned gene was sequenced by ACGT inc. (Wheeling, IL) to ensure the presence and integrity of the gene.

Mutagenesis

CaN-A447E, CaN-A451E, CaN-A454E, RD-A447E, RD-A451E, RD-A454E, and CaM-D3C were all created from the original gene in its expression vector using Stratagene QuikChange II Site-Directed Mutagenesis kit (Agilent Technologies) (Figure 2.2). RDc (Figure 2.3) was also created using materials from the Stratagene QuikChange II Site-Directed Mutagenesis kit, but the primer design and PCR was performed as follows\textsuperscript{78}: RDc was generated from the RD gene in pET303/CT-His. The deletion mutant removes
everything after the first three residues of the RD up to the three residues before the CaMBR. This is a 15 residue deletion. The primers were designed such that the reverse primer matches the N-terminus but has an overlap segment that matches the beginning of the region after the one to be deleted. The forward primer matches the segment after the region to be deleted but has an overlap segment that matches a portion of the N-terminus (before the deleted region) (Figure 2.4). The reaction mixtures were set up as the Stratagene QuikChange II Site-Directed Mutagenesis kit calls for, but the PCR was performed as in Table 2.1. All mutated genes were sequenced by ACGT inc. (Wheeling, IL) to ensure the presence of the mutations and the integrity of the genes.

**Expression and purification of recombinant CaN and CaN mutant proteins**

The CaN and CaN mutant vectors were transformed into *Escherichia coli* BL21 (DE3) CodonPlus RIL cells (Agilent Technologies, La Jolla, CA) for expression as follows: Cells containing protein plasmid were grown overnight in 100mL Terrific Broth (TB, IBI Scientific, Peosta, IA) under the selection of ampicillin (amp, 75µg/mL) at 37°C with shaking. The following day, cultures were transferred to 900mL TB/amp and grown at 37°C with shaking until OD600 reached ~1.5. Protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5mM and grown for four hours at 37°C with shaking. After expression, cells were pelleted by centrifugation at 6000g for 7 minutes at 4°C. Pellets were stored at -20°C until purification.

Cell pellets were thawed and resuspended in 1/40 of culture volume of lysis buffer (20mM Tris pH 7.5, 10mM Imidazole, 200mM NaCl, 1mM PMSF) and one Complete Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN) was added. Lysozyme was added to a final concentration of 125µg/mL and cell resuspension was incubated on ice for 20 minutes. Cells were sonicated three times for 20sec each with 3sec on, 1sec off pulse and 60% amplitude. Lysate was centrifuged at 25000g for 20min at 4°C and 0.45µm filtered before applying to a 10mL Ni-NTA column pre-equilibrated in binding buffer (20mM Tris pH 7.5, 10mM Imidazole, 200mM NaCl). Column was washed with
100mL of binding buffer and protein was eluted with 35mL elution buffer (20mM Tris pH 7.5, 2mM CaCl₂, 250mM Imidazole, 200mM NaCl). The elution was applied to a CaM-Sepharose column (GE Healthcare, Piscataway, NJ) pre-equilibrated with Tris column buffer (20mM Tris pH 7.5, 200mM NaCl, 2mM CaCl₂). The column was washed with 100mL of Tris column buffer and eluted with CaM-Seph elution buffer (20mM Tris pH 7.5, 200mM NaCl, 4mM EGTA) and collected in 15 x 1.2mL fractions. Proteins had their identities confirmed via mass spectrometry and purity confirmed by 14% SDS-PAGE analysis. If necessary, protein was dialyzed into appropriate buffer using SnakeSkin dialysis tubing (10kDa MWCO, Pierce/Thermo) and concentrated using Amicon Ultra centrifugal filter units (10kDa MWCO; Millipore, Billerica, Massachusetts). Protein concentrations were determined using either protein absorbance at 280nm or using the bicinchoninic acid assay.

**Expression and purification of recombinant RD and RD mutant proteins**

The RD and RD mutant vectors were co-transformed into *Escherichia coli* BL21 (DE3) cells with the pETCaM1 vector. CaM is co-expressed with RD or RD mutants and then removed during purification. When RD is expressed without CaM co-expression the protein is degraded in cells before purification. Cells containing protein plasmids were grown overnight in 100mL Terrific Broth (TB, IBI Scientific, Peosta, IA) under the selection of ampicillin (amp, 75µg/mL) for RD and kanamycin (kan, 50 µg/mL) for CaM at 37°C with shaking. The following day, cultures were transferred to 900mL TB/amp and grown at 37°C with shaking until OD600 reached ~1.5. Protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5mM and grown for four hours at 37°C with shaking. After expression, cells were pellet by centrifugation at 6000g for 7 minutes at 4°C. Pellets were stored at -20°C until purification.

Cell pellets were thawed and resuspended in 1/40 of culture volume of lysis buffer (20mM Tris pH 7.5, 10mM Imidazole, 200mM NaCl, 1mM PMSF) and one Complete Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN) was added. Lysozyme was
added to a final concentration of 125µg/mL and cell resuspension was incubated on ice for 20 minutes. Cells were sonicated three times for 20sec each with 3sec on, 1sec off pulse and 60% amplitude. Lysate was centrifuged at 25000g for 20min at 4°C and 0.45µm filtered. Urea/thiourea buffer (5M urea, 2M thiourea, 20mM Tris, pH 7.5, 200mM NaCl, 10mM imidazole) was added to the lysate at 3x lysate volume. The urea/thiourea is used to separate the RD from CaM, and a combination of urea and thiourea is necessary as high (8M) urea concentration alone does not separate the RD and CaM. The lysate is incubated in the urea/thiourea buffer at room temperature with stirring for 10 minutes before applying to a 10mL Ni-NTA column pre-equilibrated in urea/thiourea buffer. 50mL 5M urea/2M thiourea buffer was added to the column, and the column was incubated with shaking at room temperature for 30 minutes. The urea/thiourea concentration was decreased from 7M to 0.125M by washing the column six times with 25mL of urea/thiourea buffer that was diluted by half each time. The column was washed with 100mL of binding buffer (20mM Tris, pH 7.5, 200mM NaCl, 10mM imidazole) to remove any remaining urea/thiourea. Protein was eluted with 35mL elution buffer (20mM Tris pH 7.5, 2mM CaCl₂, 250mM Imidazole, 200mM NaCl). The elution was applied to a CaM-Sepharose column (GE Healthcare, Piscataway, NJ) pre-equilibrated with Tris column buffer (20mM Tris pH 7.5, 200mM NaCl, 2mM CaCl₂). The column was washed with 50mL of Tris column buffer and eluted with CaM-Seph elution buffer (20mM Tris pH 7.5, 200mM NaCl, 4mM EGTA) and collected in 15 x 1.2mL fractions. Proteins had their identities confirmed via mass spectrometry and purity confirmed by 14% SDS-PAGE analysis. If necessary, protein was buffer exchanged into appropriate buffer and concentrated using Amicon Ultra centrifugal filter units (3kDa MWCO; Millipore, Billerica, Massachusetts). Protein concentrations were determined using either protein absorbance at 280nm or using the bicinechonic acid assay.79
Expression and purification of recombinant CaM and CaM mutant proteins

The CaM and CaM mutant vectors were transformed into Escherichia coli BL21 (DE3) cells (Agilent Technologies, La Jolla, CA) for expression as follows: Cells containing protein plasmid were grown overnight in 100mL Terrific Broth (TB, IBI Scientific, Peosta, IA) under the selection of kanamycin (kan, 50µg/mL) at 37°C with shaking. The following day, cultures were transferred to 900mL TB/kan and grown at 37°C with shaking until OD600 reached ~1.5. Protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5mM and grown for four hours at 37°C with shaking. After expression, cells were pellet by centrifugation at 6000g for 7minutes at 4°C. Pellets were stored at -20°C until purification.

Cell pellets were thawed and resuspended in 1/40 of culture volume of CaM lysis buffer (20mM Tris pH 7.5, 2mM CaCl₂, 200mM NaCl, 1mM PMSF) and one Complete Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN) was added. Lysozyme was added to a final concentration of 125µg/mL and cell resuspension was incubated on ice for 20 minutes. Cells were sonicated three times for 20sec each with 3sec on, 1sec off pulse and 60% amplitude. Lysate was centrifuged at 25000g for 20min at 4°C and 0.45µm filtered before applying to a 10mL TAPP (2-trifluoromethyl-10-aminopropyl phenothiazine) Sepharose column pre-equilibrated with TAPP loading buffer (10mM Tris pH 7.5, 200mM NaCl, 20mM CaCl₂, 1mM β-mercaptoethanol). The TAPP-Sepharose was synthesized at the Center for Structural Biology Chemistry Core Facility, University of Kentucky (supported in part by funds from National Institutes of Health National Center for Research Resources grant P20 RR020171). The column was washed with 50mL TAPP loading buffer and the protein was eluted into 10 x 6mL fractions with TAPP elution buffer (10mM Tris-HCl pH 7.5, 200mM NaCl, 10mM EGTA). Proteins had their identities confirmed via mass spectrometry and purity confirmed by 14% SDS-PAGE analysis. Calmodulin was dialyzed into 10mM ammonium sulfate for desalting and lyophilized. Lyophilized protein was resuspended in the appropriate buffer and 0.2µm filtered. If necessary, protein was dialyzed into appropriate buffer using SnakeSkin
dialysis tubing (10kDa MWCO, Pierce/Thermo) and concentrated using Amicon Ultra centrifugal filter units (10kDa MWCO; Millipore, Billerica, Massachusetts). Protein concentrations were determined using either protein absorbance at 280nm or using the bicinchoninic acid assay.79

**Peptides**

The pCaN peptide for crystallographic studies (ARKEVIRNKIRAIGKMARVFSVLR) corresponding to the CaM binding region in the RD of αCaN was purchased from Genscript (Piscataway, NJ). pCaN peptide content was assumed to be 75% of weight and peptide resuspended in the appropriate buffer. The pCaN peptide for fluorescence studies (WGARKEVIRNKIRAIGKMARVFSVLRGGC) corresponding to the CaM binding region in the RD of αCaN was purchased from Pi Proteomics (Huntsville, AL). The N-terminal tryptophan was added to the native pCaN sequence for accurate peptide concentration determination using absorbance at 280nm and for tryptophan fluorescence measurements. The C-terminal cysteine was added for attachment of maleimide fluorophores, if necessary. The N-terminal and two C-terminal two glycines were added to act as a linker between the tryptophan or cysteine and remainder of the peptide.

Modified p-RII (WGGDLDVPIPGRFDRRV[pS]VAAE), from cAMP dependent protein kinase regulatory subunit (type II), is the CaN substrate used in the phosphatase assays and was obtained from Atlantic Peptides (Scarborough, ME).81,82 The N-terminal tryptophan was added for accurate peptide concentration determination using absorbance at 280nm, and the following two glycines were added to act as a linker between the tryptophan and remainder of the peptide. pRII was resuspended in H2O and concentration was determined by peptide absorbance at 280nm. Both peptides were purified using reverse-phase HPLC, and their identities were confirmed using mass spectrometry.
**Circular Dichroism**

CD spectra and melts were collected at 20°C using a Jasco J-810 spectropolarimeter equipped with a Peltier heating block. Protein CD spectra were obtained in buffer consisting of 20mM Tris, 200mM NaCl, and 2mM CaCl\(_2\) at a pH of 7.5. Samples containing 15µM of protein or, if a complex was being measured, 15µM of each protein were placed in a 1mm path length cuvette and CD spectra were taken. Each spectrum is an average of four scans at a scan speed of 50nm/min with five to six independent spectra taken for each sample. Errors are estimated to be no more than 3%.

CD melts were obtained using a buffer consisting of 20mM HEPES, 200mM NaCl, and 2mM CaCl\(_2\) at a pH of 7.5. Samples containing 15µM of protein or, if a complex was being measured, 15µM of each protein were placed in a 1mm path length cuvette and thermal melts were obtained with a heating rate of 1°C/min and were monitored at a wavelength of 222 nm with reported melts being the average of five or six scans.

**Fluorescence Labeling**

To create CaM-FL (D3C-CaM with Alexa Fluor 488 label) and RD-FL-C (RD with Alexa Fluor 488 label) protein to be labeled was combined with an 10x molar excess of TCEP (Tris(2-carboxyethyl)phosphine hydrochloride), pH6.8 in 20mM Tris pH 7.5, 200mM NaCl, and 2mM CaCl\(_2\) and incubated at room temperature for two hours. Alexa Fluor 488 C5 Maleimide (dissolved in water; Invitrogen, Carlsbad, CA) was added to the protein/TCEP at a 10:1 fluorescent label:dye ratio, and the mixture was diluted to a final volume of 1mL with 20mM Tris pH 7.5, 200mM NaCl, and 2mM CaCl\(_2\). Final reaction concentrations: 60µM protein, 600µM TCEP, and 600µM fluorescent label. Labeling reaction proceeded overnight, in the dark, at 4°C.

CaM-FL reaction was transferred to a 3mL, 3.5kDa MWCO Slide-A-Lyzer G2 dialysis cassette (Pierce/Thermo) and dialyzed against 2L of 20mM Tris pH 7.5, 200mM NaCl, and 2mM CaCl\(_2\) with stirring at 4°C seven times. Each dialysis proceeded for either four hours or overnight. RD reaction was was applied to a CaM-Sepharose column (GE
Healthcare, Piscataway, NJ) pre-equilibrated with Tris column buffer (20mM Tris pH 7.5, 200mM NaCl, 2mM CaCl₂). The column was washed with 50mL of Tris column buffer and eluted with CaM-Seph elution buffer (20mM Tris pH 7.5, 200mM NaCl, 4mM EGTA) and collected in 15 x 1.2mL fractions. Alexa Fluor 488 concentration was determined by absorbance at 495nm. Protein concentration was determined by absorbance at 280nm with correction for fluorescent label absorption at 280nm.

**Fluorescence Spectroscopy**

Anisotropy of RD-FL-C (Alexa Fluor 488 label), RD-FL-N (tryptophan label), and CaM-FL (Alexa Fluor 488 label) were measured using a K2 multifrequency phase fluorometer (ISS; Champaign, IL) equipped with Glan-Thompson polarizers. Anisotropies of labeled proteins were measured in 20mM Tris, pH 7.5, 200mM NaCl, 2mM CaCl₂. The anisotropies of 100nM RD-FL-C ± 300nM CaM, 100nM CaM-FL ± 300nM RD, and 100nM CaM-FL ± 300nM pCaN were measured with excitation at 495nm and emission at 520nm. The anisotropies of 10µM RD-FL-N ± 30µM CaM and 10µM pCaN ± 30µM CaM were measured with excitation at 295nm and emission at 350nM. For each protein/complex three independent samples were measured with 30 iterations for each sample.

The translational diffusion coefficients of 20nM RD-FL-C ± 100nM CaM were determined by fluorescence correlation spectroscopy (FCS) using an ALBA Fluorescence Fluctuations Spectroscopy System (ISS, USA) fitted to an inverted Nikon, Model Ti-U microscope and 488 nm and 635 nm continuous wave diode lasers. Diffusion coefficients are the averages of three independent samples. FCS was performed by Xiaolu Zhang in the Department of Chemistry at the University of Kentucky.

**Size-exclusion chromatography**

Analytical size-exclusion chromatography (SEC) was performed on RDc, CaM, and CaM:RDc using a Superdex 75 10/300 GL column on an ÄKTA FPLC both from GE Healthcare Lifesciences (Pittsburgh, PA). The running buffer used was 20mM Tris pH 7.5, 200mM NaCl, 50mM CaCl₂. All proteins were buffer exchanged into running buffer.
and 0.2µm filtered before use. Column elution was monitored at 280nm for the presence of protein. 100µL of 232µM RDc was run through the column in triplicate, and 100µL of 1.1mM CaM was run through the column in triplicate. CaM was used at a much higher concentration than RDc because CaM contains no tryptophans, but does have two tyrosines, making its absorbance at 280nm much lower than RDc which contains a tryptophan. 100µL of CaM:RDc complex was run under two different conditions: 1) excess RDc: 200µM RDc and 150µM CaM and 2) excess CaM: 150µM RDc and 200µM CaM. Gel filtration standards were purchased from Bio-Rad (Hercules, CA), and one 18mg vial was dissolved in 0.5mL running buffer, 0.2µm filtered, and 100µL run on the column.

**Structure determination**

Crystals of the CaM:pCaN complex were grown using hanging-drop vapor-diffusion experiments by Emily Holbrook, NSF-REU student. Purified CaM was combined with a slight molar excess of pCaN and concentrated to 10 mg/ml in 10mM Tris pH 7.5, 2.3mM CaCl$_2$. Protein was mixed in a 3:1 ratio of protein:mother liquor containing 24% PEG 1000, 20% glycerol in a final volume of 200 nL using a mosquito crystallization robot (TTPLabtech). Crystals formed in 2-3 weeks at 18°C. Crystals were harvested, passed through mother liquor supplemented with 10% glycerol, and flash frozen in liquid nitrogen. Diffraction data to 1.95 Å was collected at the SER-CAT ID-22 beamline of the Advanced Proton Source, Argonne National Laboratories (Table 2.2). Data were processed using HKL2000. An initial molecular replacement solution was obtained using PHASER with calmodulin N-terminal and C-terminal lobes (PDB=2W73) as the search model with the assistance of Dr. Craig Vander Kooi. Clear electron density for pCaN was observed and manually built. Iterative model building and refinement using COOT and Refmac5 by Tori Dunlap and Hou-fu Guo produced a final refined model (Table 2.2). Molecular graphics were prepared using Pymol.
Calcineurin phosphatase assays

Phosphatase assays were performed in assay buffer, 50mM Tris, pH 7.5, 100mM NaCl, 0.5mM DTT, 0.025% NP-40, 0.5mM CaCl₂ (Enzo Life Sciences, Farmingdale, NY). 50µL reactions contained 30nM CaN or mutant, 90nM CaM, and 0-800µM p-RII as substrate. Reactions were initiated with p-RII and proceeded for 7min at 30°C. Reactions were terminated and free phosphate was detected by addition of 100µL Biomol Green Reagent (Enzo Life Sciences), incubation for 20min, and absorbance measured at 620nm. Each assay was performed in five replicates. Kinetic constants were determined by fitting substrate vs initial velocity plots to the Michaelis-Menton equation using KaleidaGraph software.
Figure 2.1 Sequence of the RD construct

RD: green, CaMBR: orange, portion of AID: red, extraneous sequence: gray. Extraneous sequence was added for protein expression and purification purposes.
Figure 2.2 Sequence of the RD portion of CaN and RD mutants

The RD portion of the sequence for the following CaN or RD constructs is shown: CaN-A447E, CaN-A451E, CaN-A454E, RD-A447E, RD-A451E, RD-A454E. The underlined alanines are the amino acids that have been mutagenized to glutamates in the constructs listed. RD: green, CaMBR: orange, portion of AID: red, extraneous sequence: gray. Extraneous sequence was added for protein expression and purification purposes.
Figure 2.3 Sequence of the RDc construct

Sequence of RDc construct is shown. The sequence is the same as RD3 except there is a 15 residue deletion at the location of the arrow (the 15 residues have been removed from the sequence). RD: green, CaMBR: orange, portion of AID: red, extraneous sequence: gray. Extraneous sequence was added for protein expression and purification purposes.
Figure 2.4 Schematic of primer design to create the RDc deletion mutant

The primers were designed such that the reverse primer matches the N-terminus (core segment) but has an overlap segment that matches the beginning of the region after the one to be deleted. The forward primer matches the segment after the region to be deleted (core segment) but has an overlap segment that matches a portion of the N-terminus (before the deleted region). Melting temperature of the core segments should be ~60°C and melting temperature of the overlap segments should be ~30°C.
Table 2.1: PCR cycles for generation of the RDc mutant

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Table 2.2: Data collection and refinement statistics

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Chapter 3: Disorder to order in the regulatory domain of calcineurin

Introduction

Intrinsically disordered proteins (IDP) are those that do not possess well-defined three-dimensional structures in aqueous solution.\textsuperscript{87,88} This also includes intrinsically disordered regions (IDRs), disordered segments of polypeptide chain within an otherwise well-ordered protein. IDRs are extremely common and play crucial roles in human biology, including roles in transcription, signaling pathways, and the immune system.\textsuperscript{87,89-92} As such, IDRs are implicated in a number of human disease states such as cardiovascular disease,\textsuperscript{93} protein aggregation diseases,\textsuperscript{90} and cancer.\textsuperscript{94}

IDRs exist in conformational ensembles as, by definition, they do not have well-defined three-dimensional structure, but this does not mean that they are devoid of any structure. Disorder is a continuum from extended polypeptide chain to a molten globule state. Babu et al. have defined four types of disordered states along the disorder continuum that are useful for characterizing IDRs, from more disorder to less disorder: unstructured and extensive disorder, restricted ensemble of conformations, partially populated secondary structure and linear peptide motif, and regulated partial or complete folding upon binding.\textsuperscript{95} Where in this continuum an IDR exists is highly dependent on its sequence. In general, IDRs have a low number of hydrophobic residues and a higher polar/charged residue content as compared to well ordered proteins, but the net charge of the protein affects the nature of the disorder.\textsuperscript{87-89,92,96} Sequences enriched in polar amino acids and deficient in hydrophobic residues form compact, globular conformations, closer to the “more ordered” end of the continuum.\textsuperscript{95,97,98} As the net charge per residue increases, there is a transition from globular to loosely packed ellipsoidal coils, a “less ordered” conformation.\textsuperscript{95,99,100}

The very important calcium signaling protein calmodulin (CaM) appears to take advantage of IDRs at the “regulated partial or complete folding upon binding” end of the disorder spectrum.\textsuperscript{101} CaM has \(~300\) known binding targets\textsuperscript{66} including important enzymes such as calcineurin,\textsuperscript{73} CaM kinase I,\textsuperscript{102} and smooth muscle myosin light chain
When CaM binds a target it induces, in most but not all cases, α-helical structure in the CaM binding region (CaMBR). Radivojac et al. employed a bioinformatics approach to predict that unbound CaMBR sequences are often within disordered regions, and we have previously demonstrated that these regions often have a propensity towards α-helicity.

The serine/threonine protein phosphatase calcineurin (CaN) is regulated by CaM in this folding-upon-binding manner. CaN is important in several developmental processes as well as learning and memory, skeletal muscle growth, and immune system activation. Inappropriate CaN regulation is implicated in Alzheimer’s disease, Down syndrome, and cardiac hypertrophy. CaN is a heterodimer composed of an A-chain and a B-chain. The ~60-kDa A-chain consists of the catalytic domain, B-chain binding helix, regulatory domain (RD), an autoinhibitory domain (AID), and a C-terminal tail (CT) (Figure 3.1). The CaM binding region (CaMBR) is a 24 residue stretch within the regulatory domain. The 19-kDa B-chain is structurally homologous to CaM and binds four calcium ions. There are three dominant CaN isoforms: the neuron specific αCaN, ubiquitous βCaN, and the testes specific γCaN. Here, we discuss αCaN, and further use of CaN will refer to αCaN.

Previous proteolysis work by Yang et al. and Manalan et al., the crystal structure of CaN, and sequence analysis work all suggest that the RD may be disordered. Rumi-Masante et al. used CD spectroscopy and hydrogen-deuterium exchange mass spectrometry (HXMS) to show that a portion of CaN corresponding to the RD-AID-CT is disordered in the absence of CaM but gains α-helical structure upon CaM binding. The CaM induced structure occurs in both the CaMBR and in the region C-terminal, prior to the AID. Given that CaM seems to prefer to bind IDRs and induce folding, that the RD-AID-CT undergoes a disorder-to-order transition upon CaM binding, and that the RD-AID-CT has a low net charge per residue of ~0.04, it is likely that the RD of CaN exists in a disordered but compact, globular conformation that gains persistent secondary structure upon CaM binding.
In this work, we investigate the nature of the disorder and structure of the RD in the absence and presence of CaM. We use only the RD of CaN (instead of the RD-AID-CT) because this is the region that contains the CaMBR, and Rumi-Masante et al. demonstrated that the structure gained upon CaM binding is only in this region (Figure 3.1).\textsuperscript{73} We performed CD spectroscopy on the RD ± CaM to ensure that we see the same conformational change upon CaM binding in the RD that Rumi-Masante et al observed in the RD-AID-CT.\textsuperscript{73} Additionally, we employed fluorescence anisotropy to probe the rotational diffusion of the RD ± CaM using an RD construct with a fluorophore on the C-terminus (Alexa Fluor 488), RD-FL-C, and using an RD construct with a fluorophore on the N-terminus (tryptophan), RD-FL-N. The tryptophan added to the N-terminus of the RD is the only tryptophan in the molecule. We also measured the translational diffusion of the RD ± CaM using RD-FL-C in fluorescence correlation spectroscopy (FCS). While the RD likely exists as this compact, globular ensemble, our CD, anisotropy, and FCS experiments demonstrate that the RD undergoes further collapse upon CaM binding. CaM binding causes the RD to gain persistent α-helical secondary structure in which the RD:CaM complex has a similar hydrodynamic radius to that of the RD or CaM alone.

Results

The RD of CaN is devoid of persistent secondary structure, but gains α-helix upon CaM binding

It was shown by Rumi-Masante et. al. via circular dichroism (CD) spectroscopy and H/D exchange mass spectrometry (HXMS) that the RD-AID-CT alone is disordered, and there was no detectable gain of structure in the AID or CT upon CaM binding to the RD-AID-CT fragment.\textsuperscript{73} To analyze the secondary structure of the RD we obtained CD spectra of the RD alone. The spectrum is consistent with that of an unstructured polypeptide chain (Figure 3.2). CD spectra of CaM alone is highly α-helical, as evidenced by the strong minima at 208nm and 222nm. This is consistent with the mostly α-helical CaM seen in crystal structures.\textsuperscript{76,105-107}
When CaM is combined with an equimolar amount of a peptide corresponding to the CaMBR of CaN (pCaN) there is an increase in helical content as is expected for CaM binding to and inducing helix in a CaM-binding peptide (Figure 3.2). CaM binds to CaN with a $K_D \sim 1pM$, therefore the 15µM of both CaM and pCaN used should result in essentially all protein being bound. Upon addition of an equimolar amount of RD to CaM there is an increase in $\alpha$-helix that is greater than the increase seen with CaM:pCaN (Figure 3.2). This indicates that the RD gains $\alpha$-helix upon CaM binding, both in the CaMBR and in additional RD sequence. These data are similar to that seen by Rumi-Masante et.al. for CaM, pCaN:CaM, RD-AID-CT, and RD-AID-CT:CaM.73

**pCaN:CaM anisotropy**

The anisotropy of the tryptophan of pCaN was measured in the absence and presence of CaM (Figure 3.3a, Table 3.1). The anisotropy of pCaN alone was 0.033 ± 0.010 and increases to 0.182 ± 0.017 with the addition of CaM. This is consistent with previously reported anisotropy measurements of a tryptophan containing peptide from MLCK binding to CaM, ~0.057 for the MLCK peptide alone to ~0.180 when CaM was added.103 For comparison, the anisotropy of CaM-FL (Alexa Fluor 488 label) was measured in the absence and presence of pCaN (Figure 3.3a, Table 3.1). The anisotropy of CaM alone was 0.150 ± 0.012 and was 0.151 ± 0.002 with pCaN. The consistency of CaM anisotropy with or without pCaN is to be expected given that the increase in size that comes from binding of the 3.3kDa pCaN to the 16.7kDa CaM is small. Also, calcium loaded CaM exists as an extended conformational ensemble that typically binds by wrapping around its target peptide. This collapse of CaM around its target decreases the apparent volume of CaM which increases the rotational diffusion, decreasing anisotropy. The $K_D$ of CaM binding to CaN is $\sim 1pM$.44 With our measurements using 10µM of tryptophan fluorescent proteins, 100nM of Alexa Fluor 488 fluorescent proteins, and a 3x excess of the non-fluorescent protein in complexes, essentially all fluorescent protein should be bound in the cases where complexes are measured.
Fluorescence anisotropy is a measurement of the rotational diffusion of a fluorophore. When measuring the anisotropy of a protein labeled with a fluorophore the anisotropy is influenced by both the rotational diffusion of the protein that the fluorophore is attached to and the rotational diffusion of the fluorophore, independent of the protein. We attribute the larger anisotropy of pCaN:CaM as compared to pCaN:CaM-FL to differences in the rotational diffusion of the fluorophores, independent of the proteins. When CaM binds pCaN, pCaN forms an α-helix that is buried within CaM, and the pCaN tryptophan is at its N-terminus. Conversely, the Alexa Fluor 488 is attached to the N-terminal end of CaM which is not buried and is likely more flexible. Therefore, the pCaN tryptophan would experience less rotational freedom than the CaM Alexa Fluor 488, increasing its anisotropy.

**RD:CaM anisotropy**

The anisotropy of RD-FL-C (Alexa 488 label, C-terminus) is 0.091 ± 0.002 whereas the anisotropy of CaM-FL is 0.150 ± 0.004 (Figure 3.3b, Table 3.1). The lower RD-FL-C anisotropy is due to the smaller RD size (MW=11.8 compared to CaM MW=16.7kDa), but it also suggests that the fluorophore has more rotational freedom from being in a disordered environment, as one would expect for the disordered RD. The RD-FL-C anisotropy increases to 0.118 ± 0.008 with the addition of CaM. Comparatively, the anisotropy of CaM-FL bound to RD is 0.172 ± 0.04 (Figure 3.3b, Table 3.1). The increase in anisotropy for either protein alone (RD-FL-C:CaM = 0.027, RD:CaM-FL = 0.022) is small when one considers that the increase in size from single protein to complex is close to a doubling of size, depending on the protein being measured. This suggests that, while there is an increase in molecular weight due to the complexation of the two proteins, that increase is offset by a collapse of both molecules. For CaM, it exists in an extended conformational ensemble when not bound, and wraps around the RD, decreasing its hydrodynamic radius. The RD exists in a disordered state that collapses to a smaller hydrodynamic radius upon CaM binding. Additionally, while both anisotropies increase comparably, the anisotropy of RD-FL-C:CaM is considerably lower than that of
RD:CaM-FL. This suggests that, while binding to CaM increases the anisotropy of RD-FL-C, the fluorophore remains in a more disordered environment as compared to the fluorophore on CaM when CaM-FL binds RD.

We also measured the anisotropy of RD-FL-N (tryptophan label, N-terminus) with and without CaM as a comparison to the anisotropy of RD-FL-C with the fluorescent label on the C-terminus. The anisotropy of RD-FL-N is 0.096 ± 0.020; comparable to the 0.091 ± 0.002 of RD-FL-C. When CaM is added, the anisotropy of RD-FL-N increases to 0.149 ± 0.044 which is a considerably larger increase than RD-FL-C upon CaM addition, a 0.053 increase for RD-FL-N compared to a 0.027 increase for RD-FL-C. This suggests that the N-terminal end of the RD becomes more restricted upon CaM binding as compared to the C-terminal end of the RD. This is expected given that the N-terminal tryptophan is 20 residues from the beginning of the CaMBR whereas the C-terminal Alexa Fluor 488 label is 55 residues from the end of the CaMBR.

**Translational diffusion of RD ± CaM**

We obtained diffusion coefficients for RD-FL-C ± CaM using fluorescence correlation spectroscopy (FCS). The diffusion coefficient for RD-FL-C is 77.4 ± 0.6µm²/sec and for RD-FL-C:CaM is 74.4 ± 9.2µm²/sec (Table 3.1). The translational diffusion for both the RD-FL-C alone and complexed with CaM are essentially equivalent, within the error of the measurement. While the protein concentrations used for FCS are low, 20nM RD-FL-C ± 100nM CaM; we are confident that the RD-FL-C and CaM are bound as the $K_D$ for CaM:CaN binding is ~1pM. The equivalent translational diffusion coefficients for RD-FL-C alone and bound to CaM are consistent with our anisotropy data, suggesting that the complex, while larger in molecular weight, has a similar hydrodynamic radius to either CaM or RD alone.
Intrinsically disordered regions (IDRs) are sections of polypeptide chain within ordered proteins that do not possess stable three-dimensional structure in aqueous solution.\(^{87,88}\) However, these regions are not devoid of structure but exist as conformational ensembles whose shape can fall anywhere from extended polypeptide to molten globule. The sequence of an IDR impacts its disordered shape.\(^{95}\) IDRs that are enriched in polar amino acids but devoid in hydrophobic residues tend to form more ordered, compact, globular conformations whereas those IDRs with high a high net charge per residue form less ordered, loosely packed ellipsoidal coils.\(^{95,97-100}\) The calcium signaling protein calmodulin takes advantage of IDRs that fall on the more ordered “regulated partial or complete folding upon binding” end of the disorder spectrum.\(^{69,101}\) CaM has a tendency to bind disordered regions, wrapping around the CaMBR of the target sequence and inducing $\alpha$-helix. \(^{77,104}\) Calcineurin is one such protein that CaM binds. The CaMBR of CaN is in its regulatory domain (RD). Studies by Rumi-Masante et al. showed that the RD-AID-CT of CaN is disordered but gains $\alpha$-helix upon CaM binding in both the CaMBR and the region of the RD that is C-terminal to the CaMBR.\(^{73}\) In this work we have explored the nature of the disorder and structure in the RD of CaN with and without CaM.

We performed CD studies on the RD with and without CaM to ensure that the RD alone behaves similarly to the RD-AID-CT studied by Rumi-Masante et al., as their HXMS studies showed that the gain in structure the RD-AID-CT experiences upon CaM binding occurs only in the RD. \(^{73}\) Our CD studies demonstrated that the RD is unstructured and gains $\alpha$-helix upon CaM binding. This is consistent the work Rumi-Masante et al. performed on the RD-AID-CT.\(^{73}\) Thus, the RD does undergo a disorder-to-order transition upon CaM binding, gaining persistent $\alpha$-helical secondary structure.

We did anisotropy studies with CaM, RD, and pCaN, a peptide corresponding to the CaMBR of CaN, to investigate the rotational diffusion of the RD in its disordered state and when bound to CaM. Anisotropy studies showed a large increase in the tryptophan
anisotropy of pCaN upon CaM binding. This is expected given the binding of a 16.7 kDa protein such as CaM to a small 3.3kDa peptide like pCaN. The anisotropy change is consistent with that of CaM binding to a peptide of the CaMBR of MLCK. The anisotropy of CaM with a fluorophore bound (CaM-FL, Alexa Fluor 488), however, does not change upon binding pCaN. Canonically, CaM exists in an extended conformational ensemble that collapses and wraps around its target peptide upon binding. Therefore, upon binding pCaN, the molecular weight of the complex is not much larger than CaM-FL alone and the apparent hydrodynamic radius is smaller than that of CaM-FL alone, resulting in no change in observed anisotropy. Any change in CaM anisotropy upon RD binding would be due to the effects of the RD sequence outside of the CaMBR.

An increase in anisotropy corresponds to a decrease in rotational diffusion of the fluorophore. This can be due to an increase in hydrodynamic radius of the molecule to which the fluorophore is bound, such as when the fluorophore bound molecule binds another protein, as is the case when pCaN is bound by CaM. Alternatively, increased anisotropy can be the result of a decrease in the rotational diffusion of the fluorophore within the protein environment, such as when a fluorophore is bound to a disordered region and that region becomes structured. These two factors both impact the anisotropy of the RD in the absence and presence of CaM. We find that RD-FL-C has a lower anisotropy as compared to CaM-FL. This is partially due to the smaller size of the RD (11.7 kDa for the RD vs 16.7kDa for CaM), but is also due to the increased rotational diffusion of the fluorophore caused by the flexibility of the disordered RD. The anisotropy of CaM-FL increases upon binding RD as does the anisotropy of RD-FL-C upon binding CaM. The increases in anisotropy are comparable (RD-FL-C:CaM = 0.027, RD:CaM-FL = 0.022) and small for a change from 16.7kDa for CaM and 11.7kDa for RD to a 28.4kDa complex, suggesting that the complex has a hydrodynamic radius similar to either protein alone. However, the anisotropy of RD-FL-C bound to CaM is considerably smaller than CaM-FL bound to RD, indicating that the fluorophore on RD-FL-C still exists in a somewhat disordered environment in the RD-FL-C:CaM complex.
We also measured the tryptophan anisotropies of RD-FL-N (tryptophan label, N-terminus) with and without CaM for comparison to RD-FL-C (Alexa Fluor 488 label, C-terminus) anisotropies. The two RD species had the same anisotropy in the absence of CaM, but RD-FL-N:CaM had a larger anisotropy compared to RD-FL-C:CaM. This suggests that the tryptophan of RD-FL-N becomes more restricted upon CaM binding than the Alexa Fluor 488 on RD-FL-C does. HXMS data from Rumi-Masante et al. demonstrated that the structure in the RD gained upon CaM binding occurs both in the CaMBR and in the region C-terminal to the CaMBR.\textsuperscript{73} The structure gained in the region C-terminal to the CaMBR is an amphipathic helix termed the distal helix. It is hypothesized that the distal helix folds back onto CaM where its hydrophobic face interacts with CaM, and this hairpin-like chain reversal removes the AID from the catalytic site, activating CaN (Dunlap et al., submitted). Disruption of the hydrophobic face of the distal helix diminishes CaN activity. However, the $T_m$ for the helix is $\sim 40^\circ$C, indicating that the helix is only partially stable in \textit{in vivo}. Our RD-FL-C anisotropy data also suggest that the distal helix may be unstable as the change in anisotropy from RD-FL-C to RD-FL-C:CaM suggests that the Alexa Fluor 488 label at the C-terminal end is still in a flexible environment when CaM binds.

FCS measurements were taken to determine the translational diffusion coefficients of RD-FL-C alone and complexed with CaM. The diffusion coefficients for RD-FL-C with and without CaM were the same within the error of the experiment. This is consistent with our anisotropy studies with RD-FL-C with CaM and CaM-FL with RD. The increase in anisotropies from individual protein to complex was small, indicating little change in apparent hydrodynamic radius. The absence of significant change in the translational diffusion coefficient of RD-FL-C upon CaM binding also suggests that the hydrodynamic radius of the complex is similar to that of RD-FL-C alone. While FCS experiments are conducted at very low protein concentrations (20nM RD-FL-C ± 100nM CaM), we are confident that RD-FL-C is binding CaM under these conditions as the $K_D$ for CaM:CaN binding is $\sim 1pM$.\textsuperscript{44}
It was previously shown that the RD of CaN is disordered but gains structure upon CaM binding.\textsuperscript{73} Our CD studies have confirmed the disorder of the RD and the gain of \(\alpha\)-helix seen in the RD by Rumi-Masante et al.\textsuperscript{73} Given the low net charge per residue of the RD, one would expect it to exist as an ensemble of disordered but compact, globular conformations.\textsuperscript{95,99,100} The RD likely exists as this compact, globular ensemble, but our CD, anisotropy, and FCS experiments demonstrate that the RD undergoes further collapse upon CaM binding, gaining persistent \(\alpha\)-helical secondary structure in which the RD:CaM complex has a similar hydrodynamic radius to that of the RD or CaM alone.
Figure 3.1: Calcineurin domain structure and RD construct

a. Structure of CaN with domains highlighted. PDBID: 1AUI.52  
b. Schematic of the RD constructs used. Orange indicates the CaM binding region. The blue circle is the location of the tryptophan in RD-FL-N, and the yellow circle is the location of the Alexa Fluor 488 in RD-FL-C. The black line indicates the region of the RD that is protected in HXMS upon CaM binding as shown by Rumi-Masante et al.73
Figure 3.2: RD, CaM, pCaN CD spectra

Far UV CD spectra collected at 20°C for RD, CaM, pCaN complexed with CaM, and RD complexed with CaM.
Figure 3.3: Fluorescence anisotropies

Fluorescence anisotropy measurements for a. pCaN, pCaN + CaM, CaM-FL, and CaM-FL + pCaN; b. CaM-FL, CaM-FL + RD, RD-FL-C, RD-FL-C + CaM, RD-FL-N, RD-FL-N + CaM. Error bars indicate the standard deviation across measurements. pCaN:
peptide of the CaM binding region, CaM-FL: D3C-CaM with Alexa Fluor 488 covalently linked to C3, RD-FL-C: RD with Alexa Fluor 488 covalently linked to a cysteine at the C-terminus, RD-FL-N: RD whose anisotropy was measured using the tryptophan at the N-terminus.
Table 3.1: Anisotropies and diffusion coefficients of pCaN, CaM, RD, and complexes

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<th>Anisotropy</th>
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<td>pCaN</td>
<td>0.033 ± 0.010</td>
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<tr>
<td>pCaN + CaM</td>
<td>0.182 ± 0.017</td>
<td>-</td>
</tr>
<tr>
<td>CaM-FL</td>
<td>0.150 ± 0.012</td>
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</tr>
<tr>
<td>CaM-FL + pCaN</td>
<td>0.151 ± 0.002</td>
<td>-</td>
</tr>
<tr>
<td>CaM-FL + RD</td>
<td>0.172 ± 0.04</td>
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<tr>
<td>RD-FL-C</td>
<td>0.091 ± 0.002</td>
<td>77.4 ± 0.6</td>
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<tr>
<td>RD-FL-C + CaM</td>
<td>0.118 ± 0.008</td>
<td>74.4 ± 9.2</td>
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<tr>
<td>RD-FL-N</td>
<td>0.096 ± 0.020</td>
<td>-</td>
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<tr>
<td>RD-FL-N + CaM</td>
<td>0.149 ± 0.044</td>
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Chapter 4: Stoichiometry of the calcineurin regulatory domain-calmodulin complex

Introduction

Calcineurin (CaN) is a calcium/calmodulin (CaM) activated serine/threonine phosphatase. It was originally identified by Wang and Desai, Watterson and Vanaman, and Klee and Krinks as an inhibitor of the CaM activated cyclic 3’,5’-nucleotide phosphodiesterase. These groups demonstrated that CaN binds to, and thereby depletes, CaM in brain tissue, preventing activation of cyclic 3’,5’-nucleotide phosphodiesterase by CaM.

Since its discovery, CaN has been shown to play roles in cardiac, vasculature, and nervous system development. CaN signaling is also necessary for learning and memory, skeletal muscle growth, and immune system activation. Because of its roles in these varied signaling systems inappropriate CaN regulation has been implicated in a number of pathological states including Alzheimer’s disease, Down syndrome, and cardiac hypertrophy. Additionally, CaN’s ability to dephosphorylate and activate the nuclear factor of activated T-cells (NFAT) family of transcription factors makes it the target of the immunosuppressant drugs FK506 and cyclosporin A.

CaN is a heterodimer composed of a 57-61kDa A chain (CnA) and a 19kDa B chain (CnB). CnA is composed of the catalytic domain, CnB binding helix, regulatory domain (RD), autoinhibitory domain (AID), and C-terminal tail (CT) (Figure 4.1a). CnB is a calcium binding EF hand protein that is structurally homologous to CaM. While CnB can bind up to four calcium ions, it is constitutively bound to CnA, regardless of calcium concentrations. There are three CaN isoforms: αCaN is found primarily in neurons, βCaN is ubiquitous, and γCaN is testes specific. The pCaN and CaN regulatory domain fragment (RDC) used in this work are from αCaN. Sequence numbering and use of “CaN” refers to αCaN.
Under resting calcium conditions, CaN is maintained in an inactive state by its autoinhibitory domain (AID) bound to CaN’s catalytic site. When calcium levels rise, calmodulin (CaM) binds calcium and then binds to the calmodulin binding region (CaMBR) of CaN, located in the regulatory domain (RD) (Figure 4.1). In the absence of calcium/CaM the RD is disordered, and CaM binding to CaN causes an ordering in the RD that removes the AID from CaN’s catalytic site, activating the enzyme.

While there are several classes of sequences that CaM binds to, calcium loaded CaM preferentially binds to basic, amphipathic, $\alpha$-helical sequences (BAA sequences). In some cases, as with CaN, the CaM target sequence is disordered but prone to $\alpha$-helicity. Typically, when CaM binds BAA sequences, the CaM central linker helix becomes flexible and its two CaM lobes wrap around the target to form a compact ellipsoid (Figure 4.2 a,b).

Ye et al. have published crystal structures of CaM bound to a peptide of the CaMBR of CaN (Figure 4.1). We refer to the peptide of the CaMBR of CaN as pCaN. In these structures the CaM:pCaN complex exists as a 2:2 structure in which the N-terminal lobe of one CaM molecule and the C-terminal lobe of a second CaM molecule interact with a pCaN peptide. The remaining C- and N-terminal lobes of the two CaM molecules, respectively, interact with a second pCaN molecule (Figure 4.2c). The interaction occurs such that a pCaN peptide and two CaM lobes interact in an antiparallel fashion. The N-terminal lobe of a CaM molecule interacts with the C-terminus of pCaN, and the C-terminal lobe of a second CaM molecule interacts with the N-terminus of pCaN. While this 2:2 structure is different than the canonical “wrap-around” structure that generally occurs with calcium loaded CaM binding to a BAA sequence, the 2:2 structure does mimic a wrap-around structure when one considers only one half of the structure: an N-terminal CaM lobe and a C-terminal CaM lobe wrapped around a pCaN peptide.

Majava et al. also published a CaM:pCaN structure in which the stoichiometry is 2:2 that is very similar to the structures by Ye et al. With the high resolution of their structure (1.45Å) they are able to note that residues in both the hydrophobic pockets of CaM and
the peptide have double conformations. In addition, the authors point out that low B-
factors are seen at the exterior of the complex as compared to the interior of the complex,
suggesting that crystal contacts are what stabilize the crystal form. Majava et al. also
performed small-angle X-ray scattering (SAXS) and size-exclusion chromatography
(SEC) on the CaM:pCaN complex. The SAXS data could only be fit to a 1:1 wrap-around
model, and the SEC showed a 1:1 complex. Ye et al. performed native-gel analysis on
CaM:pCaN and SEC on a CaM:pCaN fusion protein. Both techniques showed species
corresponding to 1:1 and 2:2 molecular weights, with the 1:1 species being the dominant
form. The work by both Ye et al. and Majava et al. suggest CaM:pCaN can exist as both
a 1:1 and 2:2 complex in solution, with a 1:1 complex being the dominant species, but is
2:2 in crystal structures.

O’Donnell et al. have done extensive hydrodynamic work on CaM bound to pCaN from
βCaN (pCaNβ). The CaMBR of βCaN differs from that of αCaN by only one residue:
V395 in αCaN is I404 in βCaN. The sequence numbers are different due to an N-terminal
extension in CnA of βCaN. O’Donnell et al. used analytical gel chromatography to
determine the Stokes radii, sedimentation velocity studies to determine the sedimentation
coefficients, and NMR to determine 15N T2 relaxation times of CaM, CaM:pCaNβ, and
CaM:melittin. Melittin is a peptide that CaM is known to form a 1:1 complex with.
For each hydrodynamic experiment, CaM:pCaNβ and CaM:melittin were
identical, within the errors of the experiments. Compared to calcium-loaded CaM alone,
the CaM:peptide complexes had values consistent with a smaller hydrodynamic volume,
suggesting that, in solution, CaM:pCaNβ forms a collapsed, wrap-around 1:1 complex
similar to CaM:melittin.

In addition to hydrodynamic experiments, O’Donnell et al. used various NMR
experiments to examine some of the side-chain interactions in the CaM:pCaNβ complex.
They showed that P43 in the N-terminal lobe of CaM has an NOE with F419 at the C-
terminal end of pCaNβ, the only aromatic in the peptide. This demonstrates that, in
solution, CaM binding to pCaNβ occurs in an antiparallel fashion such that the N-
terminal lobe of CaM binds the C-terminus of the peptide and the C-terminal lobe of CaM binds the N-terminus of the peptide. All of the hydrodynamic and NMR data on CaM:pCaNβ put forth by O’Donnell et al. are consistent with a collapsed, wrap-around 1:1 structure.

Taken together, the experiments performed by Ye et al., Majava et al., and O’Donnell et al. suggest that CaM:pCaN exists as a 2:2 complex in solid-state crystallographic studies but is predominantly a 1:1 complex in solution. In this work, we attempt to reconcile the 2:2 solid-state complex and the 1:1 solution complex by doing crystallography on CaM:pCaN and analytical size-exclusion chromatography on CaM:RDc. RDc is a construct using the C-terminal portion of the RD from the beginning of the CaM binding region to the beginning of the AID (Figure 4.1b). We used CaM:pCaN for the crystallographic work so that our structure would be comparable to the previous structures, and we used CaM:RDc for the size-exclusion chromatography to determine if using a larger portion of CaN would impact the stoichiometry of CaM binding in solution.

Results

Size-Exclusion Chromatography

We performed size-exclusion chromatography (SEC) on RDc, CaM, and the CaM:RDc complex to determine the stoichiometry of the complex and to discover if the inclusion of more of CaN than just the CaMBR used in previous SEC and crystallography studies would impact the stoichiometry of the complex. In SEC runs of RDc alone and CaM alone, RDc eluted at 11.87mL, and CaM eluted at 11.60mL, where the elution volume is the volume at which the protein absorbance (280nm) peak was maximal (Figure 4.3a). Comparing SEC of RDc and CaM to the SEC standards, we can see that both proteins elute between the 44kDa standard (ovalbumin, 10.54mL) and the 17kDa standard (myoglobin, 12.28mL) which is seemingly inconsistent with their molecular weights of 10.4kDa and 16.7kDa, respectively (Figure 4.3a, Table 4.1). However, in previous work of CaM binding to a peptide of the CaM binding region of CaN (pCaN) it was shown that
the elution volume for the complex is actually lower than for that of CaM alone. This is attributed to the extended nature of calcium-loaded CaM in the absence of a binding partner. The molecular weight standards are globular in nature whereas CaM is more ellipsoidal, giving it a larger hydrodynamic radius than what would be expected for a globular protein of the same size. This is consistent with the decrease in stokes radius of CaM bound to peptide as compared to CaM alone seen by O’Donnell, et al. RDc alone also appears at an elution volume that would suggest it has a higher molecular weight than 10.4kDa. A construct of the RD-AID-CT of CaN has been shown to be disordered in solution. The RDc would also be disordered and, similarly to CaM alone, extended in conformation. Thus, RDc alone runs at a lower elution volume than expected due to its extended nature compared to the globular nature of the standards.

The absorbance of CaM at 280nm is much stronger than RDc due to the use of a much higher concentration of CaM (1.1mM CaM vs 0.232mM RD). This was done to improve the CaM absorbance at 280nm since CaM has only two tyrosines and no tryptophans. The tryptophan on RDc is extraneous sequence added to the construct for concentration determinations. Additionally, in the CaM SEC trace there is a small peak around 8mL that we attribute to protein aggregate as it elutes within the same volume as the 670kDa standard (thyroglobulin, 8.06mL) (Table 4.1).

We performed SEC on the CaM:RDc complex under conditions of excess RDc ([RDc]=200µM, [CaM]=150µM) and excess CaM ([RDc]=150µM, [CaM]=200µM) (Figure 4.3b). The complex eluted at 11.21mL under both conditions. In the complex with excess RDc SEC trace there is a peak at 11.83mL, consistent with unbound RDc. There is a shoulder on the CaM:RDc peak in the SEC trace of the complex with excess CaM that is consistent with unbound CaM, but whose elution volume could not be determined. The unbound CaM absorbance at 280nm in the excess CaM trace is much lower than the unbound RD absorbance at 280nm in the excess RD trace due to the absence of tryptophans in CaM. Additionally, in the SEC trace of the complex with excess CaM
there is a small peak around 8mL as there was in the CaM alone SEC trace. As with the CaM alone SEC trace, we attribute this peak to aggregate.

The CaM:RDc complex elutes at 11.21mL which is between the 44kDa standard (ovalbumin, 10.54mL) and the 17kDa standard (myoglobin, 12.28mL), consistent with a 1:1 complex whose molecular weight would be 27.1kDa (Figure 4.3b, Table 4.1). If the complex were 2:2 with a molecular weight of 54.2kDa the elution volume for the complex would need to be between 8.70mL and 10.54mL, the elution volumes of the 158kDa and 44kDa standards (γ-globulin and ovalbumin, respectively) (Table 4.1). There is a slight peak at ~10mL that would be consistent with a 2:2 complex, but this peak is smaller than even the aggregate seen at 8mL and much, much smaller than the 1:1 complex peak. Therefore, in our SEC studies, the CaM:RDc has a 1:1 stoichiometry.

**Crystal structure of the CaM:pCaN complex**

The CaM:pCaN complex was crystallized in 24% PEG 1000, 20% glycerol and the structure was determined at 1.95 Å. Initial model building was done using the N-terminal and C-terminal lobes of CaM from one of the CaM molecules in the 2:2 structure from Majava et al. (PDB=2W73). Crystallography data and refinement statistics can be found in Chapter 2, Table 2.2. The final structure reveals a 1:1 complex of CaM:pCaN, not a 2:2 structure like those determined by Ye et al. and Majava et al (Figure 4.2d). The difference in stoichiometry between our 1:1 structure and the 2:2 structures is likely due to the differences in crystal growing conditions. The crystals obtained by Ye et. al. were grown in 0.2M ammonium phosphate, 0.1M citrate acid (pH 4.5) and 20% (w/v) PEG 3350; and the crystals from Majava et al. were grown in 30% PEG 400, 200 mM KCl, and 100 mM Tris (pH 8.5).

The structure maintains the antiparallel binding seen in those structures as the N-terminal lobe of CaM interacts with the C-terminal end of pCaN, and the C-terminal lobe of CaM interacts with the N-terminal end of pCaN. Additionally, many of the CaM:pCaN intermolecular interactions are similar in our 1:1 structure and the 2:2 structures. I396 and
I400 of pCaN interact with M124 and F92 of CaM, respectively, in all structures (Figure 4.4). I403 of pCaN interacts with A88, V91, and V35 in CaM in all structures as well. Finally, R408 of pCaN forms salt bridges with E83 and D80 of CaM in all structures. Surprisingly, these R408:E83 and R408:D80 interactions occur between pCaN and the linker helix of CaM that is ridged in the 2:2 complexes and bent in our 1:1 complex.

Calcium-loaded CaM has two major hydrophobic pockets that aromatic residues of BAA sequences typically interact with. These are termed the FLMMn and FLMMc pockets: F19, L32, M51, and M71 in the N-terminal lobe of CaM and F92, L105, M124, and M144 in the C-terminal lobe of CaM, respectively (Figures 4.4, 4.5). In BAA sequences with one aromatic residue, the aromatic is usually buried in the FLMMc pocket, and in sequences with more than one aromatic residue one usually resides in each FLMM pocket. The only aromatic residue in pCaN is F410, and it interacts with FLMMn in the 2:2 complexes (Figure 4.5b). There are other, non-aromatic hydrophobic contacts that pCaN makes with FLMMc in the 2:2 structures (noted above), but no residues are buried in the pocket (Figure 4.4b). In our 1:1 CaM:pCaN complex F410 of pCaN is not buried in either FLMM pocket, but instead ring-stacks with P43 of CaM. O’Donnell et al (Figure 4.7) observed an NOE between F419 of pCaNβ (corresponding to F410 of pCaN) and P43 of CaM, supporting evidence for the the F410:P43 ring stacking we observe in our 1:1 complex. This NOE is not consistent with F410 binding in the FLMMn pocket.

Instead of an aromatic in either FLMM pocket, our 1:1 CaM:pCaN complex shows M406 of pCaN buried in the FLMMn pocket, contacting all of the residues of the tetrad (Figure 4.5a). This appears to be the major stabilizing interaction for CaM:pCaN in our complex. Similar to the 2:2 structures, I396 and I400 of pCaN interact with M124 and F92 of FLMMc, but no residues are buried in the pocket (Figure 4.4a).

**Discussion**

Ye, et al. and Majava et al. have determined crystal structures of CaM:pCaN that demonstrate a 2:2 complex wherein the N-terminal lobe of one CaM molecule and the C-
terminal lobe of a second CaM molecule interact with a pCaN peptide.\textsuperscript{70,74,75} The remaining C- and N-terminal lobes of the two CaM molecules, respectively, interact with a second pCaN molecule (Figure 4.2c). The interaction occurs such that a pCaN peptide and two CaM lobes interact in an antiparallel fashion. The N-terminal lobe of a CaM molecule interacts with the C-terminus of pCaN, and the C-terminal lobe of a second CaM molecule interacts with the N-terminus of pCaN. While these structures are obviously very different from the typical wrap-around binding that calcium-loaded CaM displays with BAA sequences similar to CaN’s, they do mimic the wrap-around binding in that an N-terminal lobe of CaM and a C-terminal lobe of CaM appear to wrap-around the pCaN peptide.

Majava et. al performed SAXS and SEC on CaM:pCaN in solution, finding a 1:1 complex in both cases.\textsuperscript{70} Ye et. al. also performed SEC as well as native-gel analysis on the CaM:pCaN complex and found both a 2:2 species and a 1:1 species, with the 1:1 complex being the dominant form.\textsuperscript{74,75} In addition, O’Donnell et al. did extensive hydrodynamic and NMR studies on CaM with pCaN from βCaN (pCaNβ) and found only a 1:1 complex.\textsuperscript{44} These studies, combined with the 2:2 crystal structures, suggest that CaM:pCaN exists as a 2:2 complex in solid-state crystallographic studies but is predominantly a 1:1 complex in solution.\textsuperscript{44,70,74,75} It seems that while a small population of 2:2 complexes exists in solution, the dominance of the 2:2 complex in crystal structures is likely due to the stabilizing effects of crystal contacts between complexes, as described by Majava et. al.\textsuperscript{70}

We sought to reconcile the 2:2 solid-state complexes and the 1:1 solution complexes by doing crystallography on CaM:pCaN and analytical size-exclusion chromatography on CaM:RDc, where RDc is a construct using the C-terminal portion of the RD from the beginning of the CaM binding region to the beginning of the AID (Figure 4.1b).

In both our SEC and crystallographic data we observe a 1:1 complex. In SEC, when CaM and RDc are run together, we see a peak that elutes between the volumes where the 44kDa and the 17kDa standards elute, consistent with a complex whose molecular weight
would be 27.1kDa (Figure 4.3b, Table 4.1). If the complex were 2:2 with a molecular weight of 54.2kDa the elution volume for the complex would need to be between 8.70mL and 10.54mL, the elution volumes of the 158kDa and 44kDa standards. This is similar to what Ye et al. observe in their SEC and native-gel analysis, however, we see no evidence of a 2:2 complex in our SEC. Majava et al. and O’Donnell et al. also observed only 1:1 complexes in their CaM:pCaN solution studies.44,70

We performed SEC on CaM in complex with RDc, a piece of the regulatory domain of calcineurin that is 56 residues longer than the 24 residue pCaN alone. The RDc construct was used to determine if the increased sequence space would have any impact on the stoichiometry of the complex. The extra length does not affect the complex stoichiometry, but does change the behavior of the complex in SEC. Upon addition of the pCaN peptide to CaM, Majava et al. observed a shift in the CaM SEC peak to a later elution volume than that of CaM alone. This is counterintuitive as the addition of the peptide increases the molecular weight, thereby one expects a shift to an earlier elution volume. However, CaM’s extended dumbbell shape in the absence of ligand causes it to elute at a volume consistent with a larger molecule. Therefore, when CaM wraps around the pCaN peptide, the apparent molecular weight observed by SEC decreases because its hydrodynamic radius has actually decreased.70,111

In our SEC studies, the elution volume of the CaM:RDc complex is shifted to an earlier elution volume as compared to CaM alone, as is generally the case upon an increase in molecular weight. The difference in our SEC as compared to the SEC done by Majava et al. is due to the additional residues in the RDc.

The full RD of CaN is known to be disordered in the absence of CaM but gains structure upon CaM binding.73 We used RDc, as opposed to the full RD, in our studies because Rumi-Masante et al. had shown that the structure gained in the RD upon CaM binding occurs C-terminal to the CaMBR. RDc is the majority of the RD but lacks 15 residues N-terminal to the CaMBR (Figure 4.1), a region shown by Rumi-Masante et al. not to gain structure.73 In our SEC the CaM:RDc complex elutes at an earlier volume than either
CaM or RDc alone, as expected. However, the decrease in elution time is not as much as one would expect for essentially doubling the molecular weight of the complex compared to either component alone. This suggests that, not only does CaM wrap around the RDc, causing an apparent decrease in hydrodynamic radius, but the disordered RDc also collapses upon CaM binding. We have previously shown that the α-helical structure gained in the RD upon CaM binding is an amphipathic helix that diminishes CaN activity when its hydrophobic face is disrupted. We hypothesized that, because the hydrophobic face of the helix would need another hydrophobic surface to interact with, this helix folds back upon CaM in a hairpin-like chain reversal upon CaM binding. The hairpin-like chain reversal of this helix onto CaM is consistent with our SEC results that suggest a collapse in RDc structure upon CaM binding (Dunlap et al., submitted).

In addition to our SEC studies, our crystallographic structure determination of the CaM:pCaN complex demonstrates a 1:1 stoichiometry (Figure 4.2d). Previous CaM:pCaN crystal structures have all had 2:2 stoichiometry. We have shown that a 1:1 stoichiometry also occurs in the crystallographic solid-state. Majava et al. pointed out that low B-factors are seen at the exterior of their crystal complex as compared to the interior of the complex, suggesting that crystal contacts stabilize the crystal form. In our CaM:pCaN structure the low B-factors are observed on the inside of the complex, particularly along the pCaN peptide and in the N-terminal lobe of CaM at the FLMMn residues, where M406 of pCaN is buried. Therefore, the 1:1 complex seems to be stabilized by the interaction of M406 with the FLMMn tetrad, not by crystal contacts.

While several of the intermolecular CaM:pCaN contacts are the similar between our 1:1 complex and the 2:2 complexes, there is one striking difference: the interaction of M406 of pCaN with the FLMMn pocket of CaM (Figure 4.5). This interaction is unique to the 1:1 complex not only because it is different from the 2:2 complexes, but also because it is different than other known BAA:CaM interactions. When CaM interacts with BAA sequences with one aromatic residue, the aromatic is buried in the FLMMc pocket, and in sequences with more than one aromatic residue one usually resides in each FLMM.
pocket.\textsuperscript{44,110} In pCaN, the only aromatic residue is F410. In the 2:2 complexes, F410 is buried in the FLMMn pocket, not the FLMMc pocket (Figure 4.5a). In our 1:1 complex F410 is not buried in either pocket. M406 occupies the FLMMn hydrophobic pocket, and the FLMMc pocket is unoccupied, though several small hydrophobic residues on pCaN interact with the FLMMc tetrad (Figure 4.4a). F410 ring-stacks with P43 of CaM, an interaction that is consistent with an NOE seen by O’Donnell et al. between F419 of pCaN\(\beta\) (F410 in pCaN) and P43 of CaM (Figure 4.7).

In their 2:2 crystal structure, Ye et al. observe interactions between M72 and M76 of CaM with V409 and F410 of the pCaN peptide. They also observe salt bridges between R408 of the peptide and D80 and E83 of CaM.\textsuperscript{75} These interactions occur between the peptide and the central linker helix of CaM (Figure 4.6b). In the 2:2 structures, the central linker helix is one, extended helix, allowing one CaM molecule to interact with to pCaN peptides. However, in our 1:1 structure we observe the linker helix bent as two helices with a loop region in between, allowing CaM to wrap around pCaN. Ye et al. argue that the interactions they see between pCaN and the central linker helix of CaM stabilize the extended form of the linker helix, thereby stabilizing the 2:2 structure.\textsuperscript{75} In our 1:1 structure, we do not observe the interactions between M72/M76 of CaM and V409/F410 of pCaN. The shortest distance is from M76 to V409, and that distance is \(\sim 10\text{Å}\). F410 is on the opposite side of the pCaN helix from the two CaM methionines. However, our 1:1 structure does exhibit the salt-bridges between R408 of pCaN and D80/E83 of CaM (Figure 4.6a). As these salt bridges are observed in both our 1:1 structure and the 2:2 structures, it is unlikely that they serve to stabilize the extended CaM central linker helix seen in the 2:2 structures.

From the SEC we performed on CaM:RDc and the crystal structure we determined of CaM:pCaN, we conclude that both exist as a 1:1 complex. Solution and hydrodynamic studies by Ye et al., Majava et al., and O’Donnell et al. have shown that the predominate form of CaM:pCaN in solution is the 1:1 species. Our SEC with CaM:RDc confirms this and also demonstrates that an increase in the pCaN sequence to encompass the majority
of CaN’s RD does not alter the stoichiometry of the complex. The crystal structure of CaM:pCaN we determined is also of a 1:1 complex; this is in contrast to the previously reported 2:2 CaM:pCaN structures. However, our 1:1 complex of one CaM molecule wrapping around one pCaN peptide is consistent with the NMR work and extensive hydrodynamic work done by O’Donnell et al. as well as previously published CaM:BAA structures. Our 1:1 CaM:pCaN crystal structure and SEC data for the CaM:RDc complex strongly suggest that a single CaM molecule is responsible for binding to and activating CaN.
Figure 4.1: Calcineurin domain structure and RDc construct

a. Structure of CaN with domains highlighted. PDBID: 1AUI. b. Sequence of RDc construct RD: green, CaMBR: orange, portion of AID: red, extraneous sequence: gray. Extraneous sequence was added for protein expression and purification purposes.
Figure 4.2: CaM and CaM:target peptide structures

a. Structure of calcium loaded CaM; 3CLN.76  
b. Structure of CaM bound to smMLCK CaMBR; 1CDL.104  
c. 2:2 structure of CaM bound to pCaN; 2R28  
d. 1:1 structure of CaM bound to pCaN; structure solved in this work. CaM: green, calcium: red, CaMBR peptide: blue
Figure 4.3: CaM, RDc size-exclusion chromatography

Size-exclusion chromatography traces of a. CaM alone and RDc alone and b. CaM:RDc complex in excess of RD or excess of CaM. In each graph, the vertical lines with 1, 2, 3, and 4 above them indicate the elution volume of the standards listed in Table 4.1.
Figure 4.4: FLMMc binding pocket interactions

FLMMc binding pocket interactions in a. 1:1 structure of CaM bound to pCaN; structure solved in this work. b. 2:2 structure of CaM bound to pCaN; 2R28. c. Structure of CaM bound to smMLCK CaMBR; 1CDL. CaM: green, CaMBR peptide: blue.
Figure 4.5: FLMMn binding pocket interactions

FLMMn binding pocket interactions in **a.** 1:1 structure of CaM bound to pCaN; structure solved in this work. **b.** 2:2 structure of CaM bound to pCaN; 2R28. **c.** Structure of CaM bound to smMLCK CaMBR; 1CDL. CaM: green, CaMBR peptide: blue.
Figure 4.6: pCaN R408 salt bridges with CaM D80 and E83

Salt bridge interaction between R408 of pCaN and D80, E83 of CaM in a. 1:1 structure of CaM bound to pCaN; structure solved in this work. b. 2:2 structure of CaM bound to pCaN; 2R28 75 CaM: green, CaMBR peptide: blue.
Figure 4.7: pCaN F410 and CaM P43 ring stacking

Ring stacking interaction between F410 of pCaN and P43 of CaM in the 1:1 structure of CaM bound to pCaN; structure solved in this work. CaM: green, CaMBR peptide: blue.
Table 4.1: Size exclusion chromatography standards run on the Superdex 75 10/300 GL column

<table>
<thead>
<tr>
<th>Standard</th>
<th>Molecular Weight (kDa)</th>
<th>Elution Volume (mL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Thyroglobulin (bovine)</td>
<td>670</td>
<td>8.06</td>
</tr>
<tr>
<td>2. ( \gamma )-globulin (bovine)</td>
<td>158</td>
<td>8.70</td>
</tr>
<tr>
<td>3. Ovalbumin (chicken)</td>
<td>44</td>
<td>10.54</td>
</tr>
<tr>
<td>4. Myoglobin (horse)</td>
<td>17</td>
<td>12.28</td>
</tr>
</tbody>
</table>

*Elution volume is the volume at which the protein absorbance (280nm) was maximal
Chapter 5: The distal helix in the regulatory domain of calcineurin is important for calcineurin-regulatory domain stability and calcineurin function

Introduction

The calcium signaling protein calmodulin (CaM) is important in numerous signaling pathways and is known to have approximately three-hundred binding targets. Among these targets are the calmodulin activated kinases such as CaM kinase I (CaMKI), CaM kinase II (CaMKII), CaM kinase kinase (CaMKK), and myosin light chain kinase (MLCK). CaM activated kinases possess an autoinhibitory domain (AID) that occludes the catalytic site, rendering the kinase inactive. When cellular calcium levels rise CaM binds four calcium ions and then binds the kinase at a site just C-terminal to the AID, the CaM binding region (CaMBR), causing a conformational change that removes the AID from the catalytic site, activating the kinase (Figure 5.1).

In contrast to the CaM activated kinases, there is only one known CaM activated phosphatase, the serine/threonine phosphatase calcineurin (CaN). CaN is involved in several developmental processes including formation of the cardiac, vasculature, and nervous systems. CaN is also necessary for learning and memory, skeletal muscle growth, and immune system activation. As such, inappropriate CaN regulation has been implicated in pathological states such as Alzheimer’s disease, Down syndrome, and cardiac hypertrophy. Arguably, CaN’s most well known target is the nuclear factor of activated T-cells (NFAT) family of transcription factors. CaN dephosphorylation of NFAT reveals a cryptic nuclear localization signal that allows it to move to the nucleus and initiate a gene program for the activation of T-cells. Because of this immune system involvement CaN is the target of immunosuppressant drugs. Despite its physiological importance, CaN regulation at the molecular level is still poorly understood but differs significantly from the CaM activated kinases.

CaN is a heterodimer composed of an A-chain and a B-chain. The ~60-kDa A-chain houses the catalytic domain, B-chain binding helix, regulatory domain (RD), an
autoinhibitory domain (AID), and a C-terminal tail (CT).\textsuperscript{55} The CaM binding region (CaMBR) is a 24 residue stretch within the regulatory domain (Figure 5.2). The 19-kDa B-chain is structurally homologous to CaM and binds four calcium ions.\textsuperscript{57} There are three dominant CaN isoforms: the neuron specific $\alpha$CaN, ubiquitous $\beta$CaN, and the testes specific $\gamma$CaN.\textsuperscript{58} Here, we discuss $\alpha$CaN, and further use of CaN will refer to $\alpha$CaN.

Similar to the CaM activated kinases, CaN has an AID that blocks the catalytic site until calcium-loaded CaM binds.\textsuperscript{58} However, unlike the kinases, CaN’s CaM binding region is N-terminal to the AID and separated from it by 52 residues (Figure 5.1). CaN’s CaMBR is located in the regulatory domain (RD) of CaN (Figure 5.2). Trypsin digests and other hydrolysis experiments by Manalan and Klee, and Yang and Klee, respectively, demonstrated that the RD is readily hydrolyzed, showing that it is flexible and accessible in nature.\textsuperscript{58,71} In the structure of full length CaN the electron density for the RD and C-terminal tail (CT) are missing suggesting that these regions are highly mobile.\textsuperscript{52} Additionally, Romero and Dunker noted that the sequence of the RD gave it the potential to be disordered.\textsuperscript{72} The trypsin digests performed by Manalan and Klee also suggested that the RD of CaN undergoes a large conformational change upon CaM binding as the highly unprotected RD becomes protected from trypsin digest when CaN is preincubated with CaM.\textsuperscript{71} Surprisingly, this potentially disordered RD is 95 residues long, whereas the CaM binding site is $\sim$24 residues in length, suggesting that CaM binding induces conformational changes outside of the CaMBR.

Work by Rumi-Masante et al. showed by H/D exchange mass spectrometry and circular dichroism spectroscopy that a fragment corresponding to CaN’s RD, AID, and CT (RD-AID-CT) is disordered in the absence of CaM, though the AID is well-ordered in full-length, inactive CaN.\textsuperscript{73} The RD portion of this fragment contains the CaM binding region and the 52 residues that separate it from the AID. Canonically, CaM’s two lobes wrap around its target site and induce $\alpha$-helix in that sequence. How then does CaM activate CaN with its CaMBR and AID 52 residues apart and with those 52 residues being disordered?
Rumi-Masante et al. also showed that upon CaM binding, ~50 residues within the RD become protected from H/D exchange and that the RD gains approximately fifty residues of α-helicity. About half of these residues reside in the CaMBR while the remainder are C-terminal to the CaMBR, residing between it and the AID (Figure 5.2b). The high protection and α-helicity of the CaMBR when CaM is bound is expected given the very tight binding between CaM and CaN (K_D ~1pM) and the manner in which CaM typically binds its targets. The protection and helicity in the region between the CaMBR and AID, suggested by RD protection in Manalan and Klee’s CaN/CaM trypsin digests and confirmed by Rumi-Masante et al, suggests a larger scale structuring than just the sequence CaM directly binds.

Examination of the protected area between the CaMBR and AID reveals that residues 441 to 459 could form an amphipathic α-helix (Figure 5.2c). It is unlikely that a helix in this region would be an extension of the helix formed at the CaMBR upon CaM binding as the region between the CaMBR and this proposed helix is populated with glycines and prolines, residues known to be unfavorable in α-helices (Figure 5.2b). An amphipathic helix in this region would imply the need for the hydrophobic face of the helix to interact with another hydrophobic region, however we know from Rumi-Masante et al. that CaM bound RD-AID-CT does not interact with the remainder of CaN and that the only regions in the RD-AID-CT to gain significant secondary structure upon CaM binding is the CaMBR and part of the region between it and the AID. Therefore, we hypothesize that an amphipathic helix in the region between the CaMBR and AID would interact with the surface of the remainder of the RD:CaM complex when CaM is bound to the CaMBR of CaN. The end of this proposed helix is seven residues from the beginning of the AID. If it forms and folds back upon RD:CaM when CaM binds the RD, this could provide the necessary energy to remove the AID from the catalytic site. We will refer to this possible helix as the distal helix. In this work, we explore the nature and importance of the structure and function of this putative distal helix in the RD of CaN.
Results

*RD:CaM secondary structure is similar to that of the RD-AID-CT:CaM*

To investigate CaM induced structure and a possible distal helix in the region C-terminal to the CaMBR we created a construct consisting of only the RD of CaN (Figure 5.2b). It was shown by Rumi-Masante et. al. via circular dichroism (CD) spectroscopy and H/D exchange mass spectrometry (HXMS) that there was no detectable gain of structure in the AID or CT upon CaM binding to the RD-AID-CT fragment. To analyze the secondary structure of the RD we obtained CD spectra of the RD alone. The spectrum is consistent with that of an unstructured polypeptide chain (Figure 5.3a).

An equimolar mixture of CaM and a 24-residue peptide corresponding to the CaMBR of CaN (pCaN) yields a spectrum with increased α-helix as compared to CaM alone, as is evident from the more negative minima of CaM:pCaN at 208nm and 222nm, indicators of α-helix (Figure 5.3a). This increase in helicity is expected for CaM binding and inducing helix in its target. However, an equimolar mixture of CaM and the RD demonstrates that the RD:CaM complex has greater helical content than the pCaN:CaM complex (Figure 5.3a). These data are similar to that seen by Rumi-Masante et.al. for CaM, pCaN:CaM, RD-AID-CT, and RD-AID-CT:CaM.

*CaM induced RD structure is less stable than pCaN:CaM*

Thermal melts of pCaN:CaM and RD:CaM from 10°C to 95°C were obtained monitoring CD at 222nm, the wavelength associated with minima in CD spectra caused by α-helices. The pCaN:CaM melt reveals no melting transitions, indicating that the pCaN:CaM complex is stable to at least 95°C. The melt of RD:CaM, however, shows a melting transition with Tm~40°C (Figure 5.3b). The major sequence difference between pCaN:CaM and RD:CaM lies with the addition of the region C-terminal to the CaMBR (Figure 5.2b), thus the melting transition seen in RD:CaM is consistent with the melting of a helix in this region.
Disruption of the hydrophobic face of the putative distal helix alters CaM induced secondary structure

The hydrophobic face of the putative distal helix is composed of a leucine, isoleucine, and three alanines (Figure 5.2c). We created three RD mutants, RD-A447E, RD-A451E, and RD-A454E, that would disrupt the hydrophobic face but not necessarily prevent the formation of a helix as glutamates are charged but have high helical propensities. The CD spectra of these mutants demonstrate that they are unstructured polypeptide chains similar to wild-type RD (Figure 5.4a).

In contrast, addition of equimolar CaM to the RD mutants yields CD spectra that have helical content more similar to that of pCaN:CaM, not RD:CaM (Figure 5.4b). The similarity of the RD mutant:CaM spectra to the pCaN:CaM spectrum indicates that secondary structure in the region between C-terminal to the CaMBR has been disrupted by the alanine to glutamate mutations. However, the RD mutant:CaM spectra have slightly stronger 222nm minima than pCaN:CaM, with RD-A454E being the strongest, suggesting that they may have some residual distal helix.

Disruption of the hydrophobic face of the putative distal helix alters CaM induced helix stability

Thermal melts of the RD mutants RD-A447E, RD-A451E, and RD-A454E, with CaM were obtained, monitoring the CD at 222nm from 10°C to 95°C as for pCaN:CaM and RD:CaM above. Consistent with the CD spectra (Figure 5.4b), the RD mutants with CaM have more strongly negative signals than pCaN:CaM at the beginning of the melt, with RD-A454E being the strongest (Figure 5.4c). This is indicative of more α-helix in the mutants with CaM than pCaN:CaM. However, all the mutants have signals approaching that of pCaN by the end of the melts. Wild-type RD:CaM has a stronger helical signal than any of the mutants with CaM or pCaN:CaM, demonstrating its increased helicity in the region C-terminal to the CaMBR. Despite its weakened helical signal as compared to RD:CaM, RD-A454E:CaM does have a melting transition. The T_m for this melting could not be obtained due to the absence of a native baseline for the RD-A454E:CaM melt. The
weaker helical signal of RD-A454E:CaM compared to RD:CaM combined with the presence of a melting transition suggests that the RD-A454E retains a partial distal helix, more than that of the other mutants, but the helix is diminished as compared to wild-type RD.

**Disruption of the hydrophobic face of the putative distal helix diminishes CaM activated CaN activity**

To investigate the impact of the disruption of the hydrophobic face of the putative distal helix on CaN dephosphorylation kinetics we created the same three alanine to glutamate mutations in full-length CaN as we did in the RD: CaN-A447E, CaN-A451E, and CaN-A454E. The CaM induced activity of these mutants, as well as wild-type CaN, was assessed with a malachite green free phosphate assay using varying concentrations of p-RII peptide from cAMP dependent protein kinase regulatory subunit (type II), a well characterized CaN substrate. Reactions were performed with 1:3 CaN:CaM or CaN mutant:CaM. Various divalent cations are known to impact CaN’s activity in different ways. In our assays we include calcium and magnesium. Table 1 gives the kinetic parameters for CaN and the CaN mutants obtained under the conditions of our assay. This kinetic analysis of CaN and CaN mutant dephosphorylation activity reveals increased $K_m$ values for the CaN mutants as compared to wild-type CaN (Table 1). The increased $K_m$ values demonstrate that the integrity of the hydrophobic face of this putative helix is both important for CaN activity and also impacts CaN’s ability to bind substrate.

**Discussion**

In CaM activated kinases the CaMBR is directly C-terminal to the AID (Figure 5.1). This proximity allows CaM to bind, causing the CaMBR to form an α-helix which removes the AID from the catalytic site, activating the kinase. Despite CaN having both a CaMBR and AID as the CaM activated kinases do, CaM activation of CaN cannot occur in the same way as the CaM activated kinases given that the C-terminal end of the CaMBR is 52 residues N-terminal to the AID and these residues are disordered in the
absence of CaM (Figure 5.1). The intervening disordered residues seemingly prevent the removal of the AID from the active site when CaM binds at CaN’s CaMBR.

Proteolytic digest work by Manalan and Klee and Yang and Klee showing that the RD is readily degraded by proteases; missing RD and CT electron density in the CaN structure; and sequence analysis by Romero and Dunker all suggested that the RD is disordered. Additionally, Manalan and Klee’s trypsin digests of CaN preincubated with CaM showed that the RD became protected when CaM was bound to CaN, suggesting that the RD undergoes a conformational change upon CaM binding. Rumi-Masante, et al showed that the RD-AID-CT is disordered but gains structure upon CaM binding. This structure includes the expected \( \alpha \)-helix in the CaMBR, but it also includes one or more regions of helical structure somewhere between the end of the CaMBR and the beginning of the AID.

In this work, we investigated a construct of the RD alone. Our CD spectra for RD and RD:CaM are comparable to those for RD-AID-CT and RD-AID-CT:CaM done by Rumi-Masante, et al, confirming that the \( \alpha \)-helix gained upon CaM binding is in the RD, not the AID or CT. We also performed thermal melts of pCaN:CaM and RD:CaM. The thermal melt of pCaN:CaM shows no cooperative melting transition as pCaN:CaM is extremely stable (\( K_D \) for CaN:CaM is \( \sim \)1pM) and does not melt within the 10°C to 95°C range of our thermal melts. Therefore, the melting transition seen in the RD:CaM thermal melt must come from melting of helical structure in the RD outside of the CaMBR. Thus, CaM induced helical content is both in the CaMBR and the remainder of the RD (Figure 5.3).

Upon examination of the sequence in the region of the RD C-terminal to the CaMBR, identified by Rumi-Masante et al as the region besides the CaMBR that gains structure upon CaM binding, we identified a portion that plots as an amphipathic helix on a helical wheel projection (Figure 5.2c). From this we proposed that an amphipathic helix in this region forms by folding its hydrophobic face onto the surface of the rest of the RD:CaM complex when CaM is bound to the CaMBR of CaN. We refer to this helix as the distal
helix. Rumi-Masante et al showed that CaM bound RD-AID-CT does not interact with CaN’s catalytic domain, B-chain binding helix, or B-chain, making the remainder of the RD:CaM complex the only other portion of CaN that the distal helix could interact with. Interaction of the distal helix with the remainder of the RD:CaM complex would create a hairpin-like chain reversal that could compensate for the 52 residues between the CaMBR and AID allowing for the removal of the AID from the catalytic site, activating CaN. This helix would not be an extension of the CaMBR helix as the region between the CaMBR and this helix contains multiple glycines and prolines, residues known to be unfavorable in α-helices.

To test for the presence of the distal helix we created three RD mutants: RD-A447E, RD-A451E, and RD-A454E (Figure 5.2). The alanine to glutamate mutations would disrupt the hydrophobic face of the helix but not necessarily prevent the formation of a helix as glutamates are charged but have high helical propensities. CD spectra of these mutants were comparable to wild-type RD showing that they are disordered, but upon CaM binding the helical content gained is similar to that of pCaN:CaM, not RD:CaM (Figure 5.4a,b). This indicates that the mutations have caused a reduction in CaM-induced helical content.

Thermal melts of the RD mutants with CaM were similar to the thermal melt of pCaN:CaM, demonstrating that the lost helical content is in the RD, but outside of the CaMBR (Figure 5.4c). Though the CD spectra and melts are closer to pCaN:CaM than RD:CaM, the RD-A454E:CaM and RD-A447E:CaM complexes shows more residual helical content than RD-A451E:CaM. RD-A454E:CaM even has a visible melting transition, though a $T_m$ could not be determined. As A451 is central to the distal helix, and A447 and A454 would be nearer the N- and C-terminal limits of the helix, mutation of the central alanine (A451) may disrupt the entire helix whereas mutation of the alanines closer to the termini (A447 and A454) may still allow for partial formation of the distal helix. We also note that thermal melting of the RD showed that the distal helix’s $T_m$ is ~40°C, very near physiological body temperature of 37°C, suggesting that it is not very
stable. Given this, the reduced helical content of the hydrophobic face mutants, and the absence of this helix when CaM is not bound to CaN suggests that the helix requires that its hydrophobic face interact with the remainder of the RD:CaM complex in order for it to form.

To investigate the importance of the distal helix in CaN dephosphorylation activity, we created the same mutations in full-length CaN as we did in the RD: CaN-A447E, CaN-A451E, and CaN-A454E. Performing malachite green free phosphate assays on CaM activated wild-type CaN and these mutants revealed increased $K_m$ in the mutant CaNs as compared to wild-type (Table 1). The increased $K_m$s demonstrate a reduced affinity for substrate in the mutant CaNs. This alteration in kinetic parameters shows that the proper formation of the distal helix is important for CaN dephosphorylation, but it is of note that the mutants diminish CaN activity, they do not abolish it. The increased mutant CaN $K_m$s suggest that the AID can still bind the active site in these mutants, even in the presence of CaM. However, the residual activity in these mutants suggests that in the CaN mutants with CaM the AID binding in the active site is not as tight as it is in wild-type CaN without CaM, likely due to residual distal helix in the mutants.

Based on our alanine mutation studies we have shown that the distal helix is comprised of at least eight residues if A447 and A454 are considered to be the bounds of the helix. However, trypsin digest work by Rumi-Masante et al shows that both K441 and K459 are protected from digestion, suggesting that the distal helix may extend from residue 441 to 459, beyond the alanine limits (Figure 5.2b). This gives an ~19 residue helix, which is consistent with the helical gain seen in the region between the CaMBR and AID by Rumi-Masante et al. This is also consistent with our helical wheel projection (Figure 5.2c).

We have shown that disruption of the hydrophobic face of the putative distal helix diminishes the helical content in this region and reduces CaN’s affinity for substrate. Residual CaN activity when the hydrophobic face of the distal helix is disrupted may be due to residual helicity of the distal helix. In light of the importance of the distal helix for...
CaN activity, we propose the following model for CaN activation by CaM: When CaM binds to the CaMBR in the RD of CaN a stretch of residues N-terminal to the AID forms an amphipathic α-helix, which we have termed the distal helix. The hydrophobic face of the distal helix must interact with another hydrophobic region on the remainder of the RD:CaM complex to form. This interaction creates a hairpin-like chain reversal in the RD that compensates for the 52 residues between the CaMBR and AID, and allows the AID to be removed from CaN’s catalytic site when CaM binds, activating CaN.
Figure 5.1: Domain ordering of CaM activated kinases compared to the domain ordering of CaN

In CaM activated kinases, the AID is just N-terminal to the CaM binding domain, allowing for easy removal of the AID from the kinase active site upon CaM binding. In CaN, the AID is 52 residues C-terminal to the end of CaM binding domain suggesting a different mode of CaM activation in CaN from the kinases.
Figure 5.2: Calcineurin domain structure and regulatory domain schematics

**a.** Structure of CaN with domains highlighted. Catalytic domain: purple; CnB binding helix: blue; Regulatory domain (RD): green, drawn, electron density is missing from crystal structure; CaM binding region: orange rectangle, drawn, electron density is missing from crystal structure; Autoinhibitory domain (AID): red; C-terminal tail (CT): light gray, drawn, electron density is missing from crystal structure; Calcineurin B (CnB): dark gray. PDBID:1AUI.52  

**b.** Sequence of RD construct with distal helical region highlighted by the blue region and the mutated alanine residues underlined. RD: green, CaMBR: orange, portion of AID: red, extraneous sequence: gray. Extraneous sequence was added for protein expression and purification purposes.  

**c.** Helical wheel projection of the distal helix. The alanine to glutamate mutations made on the hydrophobic face are indicated. N and C mark the N-terminal and C-terminal residues of the putative helix, respectively.
Figure 5.3: CD spectra and melts of RD, CaM, pCaN, and complexes

a. Far UV CD spectra collected at 20°C for RD, CaM, pCaN complexed with CaM, and RD complexed with CaM. b. Thermal melts following CD signal at 222nm from 10°C to 95°C for pCaN complexed with CaM and RD complexed with CaM.
Figure 5.4: CD spectra and melts of RD mutants and RD mutant:CaM complexes

Far UV CD spectra collected at 20°C for a. RD, RD-A447E, RD-A451E, and RD-A454E as well as b. the following, each complexed with CaM: pCaN, RD, RD-A447E, RD-A451E, RD-A454E. c. Thermal melts following CD signal at 222nm from 10°C to 95°C for each of the following complexed with CaM: pCaN, RD, RD-A447E, RD-A451E, RD-A454E.
Table 5.1: Kinetic parameters of CaN and CaN distal helix mutants for dephosphorylation of p-RII peptide

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (umol min$^{-1}$ mg$^{-1}$)</th>
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<tbody>
<tr>
<td>CaN</td>
<td>464 ± 60</td>
<td>12.0 ± 0.9</td>
</tr>
<tr>
<td>CaN-A447E</td>
<td>478 ± 114</td>
<td>8.7 ± 1.2</td>
</tr>
<tr>
<td>CaN-A451E</td>
<td>899 ± 213</td>
<td>9.7 ± 1.5</td>
</tr>
<tr>
<td>CaN-A454E</td>
<td>589 ± 101</td>
<td>11.1 ± 1.1</td>
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Chapter 6: Conclusions and future directions, a model for calcium/CaM regulation of CaN

Calcineurin (CaN) signaling is vitally important for the fetal development of numerous tissues, necessary for T-cell activation, and important in learning and memory. Not only is CaN important in human physiology, but it is highly conserved and necessary for survival from yeast to humans.\(^4\) CaN signaling is very well studied, largely due to CaN’s role in the activation of the NFAT (nuclear factor of activated T-cells) family of transcription factors by dephosphorylation. Because NFAT signaling in T-cells leads to activation of T-cells, CaN has become the target of immunosuppresant drugs.\(^4,5\)

CaN is a heterodimer composed of a 57-61kDa A chain (CnA) and a 19kDa B chain (CnB). CnA is composed of the catalytic domain, CnB binding helix (CnBBH), regulatory domain (RD), autoinhibitory domain (AID), and C-terminal tail (CT) (Figure 1.3).\(^55\) It is known that at low calcium concentrations the AID resides in the catalytic site of CnA, keeping the enzyme inactive. When calcium levels rise, calcium loaded CaM binds to the CaM binding region (CaMBR) located in the regulatory domain of CnA. CaM binding releases the AID from the catalytic site, activating CaN.\(^56\) CnB is a calcium binding EF hand protein that is structurally homologous to CaM and is constitutively bound to CnA, regardless of calcium concentrations.\(^57\)

Despite being important and highly studied, there is much we do not know about the protein CaN. The efforts in this body of work have focused on furthering our understanding of the physical aspects of calmodulin (CaM) regulation of CaN. The work of many who have come before us has shown that the isolated regulatory domain is disordered in the absence of CaM but undergoes a conformational change upon CaM binding that induces α-helical structure in the RD and, in full-length CaN, removes the AID from CaN’s catalytic site to activate the enzyme.\(^52,71-73,115\) This knowledge brings with it more questions.

A disordered protein or protein region is not devoid of all structure but exists as a conformational ensemble that can be anywhere from loosely packed coils to compact and
globular. To describe the RD of CaN as disordered only puts it into this wide spectrum. Thus, we sought to discover the nature of the disorder in the RD and the change in that disorder upon CaM binding (Chapter 3). We examined this by investigating the rotational and translational diffusions of the RD ± CaM using fluorescence anisotropy and fluorescence correlation spectroscopy, respectively. Our studies have shown that, given its low net charge per residue, the RD likely exists as this compact, globular ensemble, but CD, anisotropy, and FCS experiments demonstrate that the RD undergoes further collapse upon CaM binding, gaining persistent α-helical secondary structure in which the RD:CaM complex has a similar hydrodynamic radius to that of the RD or CaM alone. However, a comparison of the anisotropy of RD with a label on the C-terminus vs the N-terminus, with CaM, suggested that C-terminus of the RD remains at least somewhat flexible and disordered upon CaM binding. Given what we later learned about the existence of the distal helix (Chapter 5) C-terminal to the CaMBR, that it is important for efficient CaN activity but only partially stable with a T_m of ~40°C, we believe that the apparent partial disorder remaining at the C-terminus of the RD upon CaM binding is likely the result of transient folding and unfolding of this helix. However, we note that the T_m of the helix in vivo may be higher due to the crowding conditions found in cells.

It was also noted that there remained uncertainty concerning the stoichiometry of the CaN:CaM complex. Ye et al. and Majava et al. have published crystal structures that demonstrate a 2:2 complex of a peptide corresponding to the CaMBR of CaN (pCaN) and CaM. However, solution work by these groups as well as extensive hydrodynamic and NMR studies by O’Donnell et al. suggested the dominant stoichiometry in solution is 1:1, with only a minor fraction existing as a 2:2 complex when the 2:2 complex was seen at all.

Without a confirmed knowledge of the stoichiometry of the CaM:CaN complex, any attempt to understand the mechanism by which CaM activates CaN would be incomplete. We attempted to reconcile the 2:2 solid-state complex and the 1:1 solution complex by using x-ray crystallography to solve the structure of the CaM:pCaN complex and
analytical size-exclusion chromatography (SEC) on CaM:RDC, with RDC being a construct of the C-terminal portion of the RD from the beginning of the CaM binding region to the beginning of the AID. Both the SEC studies and our crystal structure demonstrated a 1:1 complex, with no observable 2:2 complex present. The 1:1 complex of one CaM molecule wrapping around one pCaN peptide is consistent with the NMR work and extensive hydrodynamic work done by O'Donnell et al. as well as previously published structures of CaM binding to other basic, amphipathic α-helical peptides of the same CaM binding target class as pCaN.\textsuperscript{44,110} Our 1:1 CaM:pCaN crystal structure and SEC data for the CaM:RDC complex strongly suggest that a single CaM molecule is responsible for binding to and activating CaN.

We know now that CaM binds to a disordered RD with 1:1 stoichiometry, wraps around the CaMBR, and causes a conformational ordering in the RD that removes the AID from CaN’s catalytic site, activating the enzyme. So, how does the ordering that CaM induces in the RD remove the AID from CaN’s active site? There are 52 residues from the end of the CaMBR to the beginning of the AID. If CaM bound the RD and only induced α-helix in the CaMBR, 52 residues would be plenty of disordered sequence space to allow the AID to remain bound in the active site. However, Rumi-Masante et al. showed that ~25 residues in this region gain α-helical structure upon CaM binding.\textsuperscript{73}

Examination of the protected area between the CaMBR and AID reveals that residues 441 to 459 could form an amphipathic α-helix (Chapter 5, Figure 5.2). It would be unlikely for this to be an extension of the CaMBR helix as the intervening sequence is populated with glycines and prolines which are unfavorable in helices.\textsuperscript{114} An amphipathic helix in this region would imply the need for the hydrophobic face of the helix to interact with another hydrophobic region, and we hypothesized an amphipathic helix in the region between the CaMBR and AID would interact with the surface of the remainder of the RD:CaM complex when CaM is bound to the CaMBR of CaN. The end of this proposed helix is seven residues from the beginning of the AID. If it were to form and fold back
onto the surface of CaM in a hairpin-like chain reversal, it could provide the force necessary to remove the AID from the active site.

We explored the possibility of this helix and its function in CaN activity by creating mutations in the isolated RD and full-length CaN that would disrupt the hydrophobic face of the helix. We obtained CD spectra and melts of the WT-RD and mutants ± CaM, as well as performed CaN phosphatase assays on WT-CaN and mutants in the presence of CaM. Our work demonstrated that disruption of the hydrophobic face of what we termed the distal helix diminishes the helical content in this region and reduces CaN’s affinity for substrate. Residual CaN activity when the hydrophobic face of the distal helix is disrupted may be due to residual helicity of the distal helix.

Our work has demonstrated that CaM binds to a disordered RD with 1:1 stoichiometry and causes a compaction of the RD that includes the formation of the distal helix, an element important for efficient CaN activity. From this the question arises: Why does the distal helix exist? In lower eukaryotes, such as *drosophila* and yeast species, the distal helix sequence is absent (Figure 6.1). It seems that the absence of the sequence for the distal helix would allow for the direct removal of the AID from CaN’s active site without the need for the hairpin-like chain reversal that the distal helix causes. CaM binding to and inducing α-helix in the CaMBR may alone provide the steric force necessary to remove the AID in the absence of the distal helix. *Drosophila* CaN is very similar to human CaN both in sequence and length, but in *drosophila* the helical sequence is missing, possibly allowing for direct removal of the AID. Many yeast species also have a deletion of the distal helix sequence, however they also have an insertion in the RD, N-terminal to the CaMBR (Figure 6.1). In *Aspergillus fumigatus* the insertion has several serine-proline motifs, the phosphorylation of which is crucial for appropriate CaN signaling (Juvvadi, P.R., et al., in revision).

It seems that the variations in the RD amongst species are added levels of CaN regulation. In *Aspergillus fumigatus* phosphorylation in the RD regulates CaN signaling, and in *drosophila* the distal helix is missing, possibly allowing for quicker, more efficient
activation of CaN. So, if these variations are regulatory elements, what is the regulatory function of the distal helix in humans? The answer may lie in the calcium binding abilities of CnB.

CnB is the calcium binding subunit of CaN. It is structurally homologous to CaN with four calcium binding EF-hands. CnB binds CnA in an antiparallel manner where the C-terminal lobe of CnB binds to the N-terminal end of the CnB binding helix (CnBBH) and forms an interface with the catalytic domain of CnA. The N-terminal lobe of CnB binds to the C-terminal end of the CnBBH. The C-terminal lobe EF-hands are the high affinity calcium binding sites with $K_D \sim 70\text{nM}$. At low calcium conditions comparable to resting cellular concentrations, the C-terminal lobe of CnB is calcium loaded and bound to CnA. However, the N-terminal lobe of CnB would be devoid of calcium and partial proteolytic digests suggest that, in the absence of calcium binding, the N-terminal lobe does not bind the CnBBH. Furthermore, these partial proteolytic digests also show that the RD in CaN is protected from digest in the absence of calcium but is rapidly degraded when calcium is present. This suggests that a portion of the RD folds onto CnB or the CnBBH in the absence of calcium, and calcium binding to the N-terminal lobe of CnB causes it to bind the CnBBH, releasing the RD.

Brian Perrino, who is now at the University of Nevada, Reno, created a construct corresponding to the RD from the end of the CaMBR to the end of the C-terminal tale, encompassing the distal helix and AID, AI420-511. When assayed with constitutively active CaN (CaN1-420, missing the distal helix and AID-CT) this construct is an ~five-fold better inhibitor of CaN than the AID alone. Full activation of CaN requires both calcium binding to CnB and calcium-loaded CaM binding to the RD. Calcium binding to the CnB results in a lowered $K_m$ while CaM binding increases the $V_{\text{max}}$. Addition of calcium to CaN (in the absence of CaM) lowers the $K_m$ ~three-fold. Our hypothesis is that a portion of the RD including the distal helix interacts with CnB/CnBBH in the absence of calcium and is released upon calcium binding. The interaction of the distal
helix region with CnB/CnBBH likely blocks substrate access to the LxVP docking site that is located at the CnB/CnA interface. Release of the distal helix from CnB/CnBBH would decrease the Km of CaN (increasing CaN’s substrate affinity) by exposing the LxVP docking site, allowing substrate to bind. We do not discount the role of CnB in propagating a conformational change to the catalytic domain upon calcium binding. It is likely that this occurs in addition to the changes in the RD, as lower eukaryotes without the distal helix still require CnB for CaN function. Thus, it seems the distal helix may play a calcium sensitive regulatory role in allowing substrate binding to the LxVP docking site.

However, this does not fully explain the role of the distal helix in CaN regulation as we have seen that *drosophila* are missing the distal helix and have no RD insertion that could compensate for the missing distal helix. Why does *drosophila* not need the regulation provided by a distal helix, as seen in humans, or an N-terminal RD insertion, as seen in yeast? The answer is not readily apparent, but could be addressed by examining the activity of wild-type *drosophila* CaN as well as *drosophila* CaN with a distal helix insertion or an N-terminal RD insertion, both in vitro and in cellular environments. We also note that the LxVP docking site that we believe the distal helix blocks under low calcium conditions is not the only substrate docking site. The PxIxIT motif present in NFAT substrates as well others docks on CnA, opposite from the LxVP docking site (Chapter 1, Figure 1.2). LxVP docking seems to be important for substrate recognition, but the use of the PxIxIT docking site may allow for some substrates to bind CaN even in low calcium conditions, giving them ready access to the LxVP docking site when it becomes available.\(^{21}\)

Our current model for calcium/CaM activation of CaN is: at resting cellular calcium levels the N-terminal lobe of CnB is devoid of calcium and not bound to the CnBBH. This allows the distal helix region of the RD to interact with CnB/CnBBH, occluding the LxVP substrate docking site. When calcium levels increase, the RD is released from its interaction with CnB/CnBBH by the N-terminal lobe of CnB binding calcium and then
the CnBBH. Release of the RD from CnB/CnBBH exposes the LxVP site, allowing for substrate docking at this site. Calcium-loaded CaM then binds the now disordered RD, causing formation of the distal helix which folds upon CaM in a hairpin-like chain reversal. The formation and chain reversal of the distal helix removes the AID from CaN’s catalytic site, causing the full activation of CaN (Figure 6.2).

Calcium binds to CaM with $K_D$ values of ~0.2$\mu$M and ~2$\mu$M at the C-terminal and N-terminal lobes, respectively.\(^{117-119}\) The N-terminal lobe of CnB has a $K_D$ of calcium binding of ~20$\mu$M.\(^{60}\) Thus, even the low affinity calcium binding N-terminal lobe of CaM has a 10-fold higher calcium affinity than the N-terminal lobe of CnB. This suggests that when the CnB N-terminal lobe binds calcium and then the CnBBH, releasing the RD, CaM is already calcium loaded and ready to bind the RD as soon as it is available. While disordered proteins and regions have been shown to exist, and it has been proven that the disorder is often necessary for a protein’s function, it remains a mystery as to how disordered proteins and regions survive a cellular environment that is designed to degrade and dispose of misfolded and unfolded proteins. The system of RD disorder and CaM binding may provide insight. If CaM is calcium loaded and ready to bind the RD as soon as it is released from the CnB/CnBBH, then the disorder of the RD is very transient. The disorder of the RD is important to allow CaM binding, but the disorder exists only for a short time before CaM binding, especially given that the $K_D$ of calcium loaded CaM binding to CaN is ~1pM.\(^{44}\) Before calcium binding to the CnB, the RD is protected by interaction with the CnB/CnBBH, and as soon as it is released CaM binds, and it is protected by interaction with CaM. Thus, transient disorder may be one mechanism by which the necessary disorder of some proteins is controlled in cellular environments.

Our new knowledge of the disordered nature of the isolated RD and the formation and possible regulatory function of the distal helix has significantly contributed to our understanding of CaM activation of CaN, but it also raises new questions. At low calcium levels, we hypothesize the distal helix region of the RD binds to CnB/CnBBH, occluding the LxVP substrate binding site. We can investigate this possibility by obtaining a
fluorescently labeled peptide corresponding to the distal helix region of the RD and measure the fluorescence anisotropy of the peptide with and without CaN373, a construct that includes CnA up to the end of the CnBBH and CnB. This must be done in the absence of calcium. If the peptide binds CaN373, it may be possible to obtain an anisotropy binding curve to determine the affinity of binding. We can mutate residues in the CnBBH and LxVP docking site and measure the anisotropy of binding of the distal helix peptide to these mutants for comparison to the binding of peptide to CaN373. Additionally, a fluorescently labeled peptide of a substrate LxVP motif could be obtained and its anisotropy measured with/without CaN373 and the distal helix peptide. If the distal helix peptide blocks the LxVP docking site, the LxVP motif peptide will not be able to bind CaN373 in its presence without calcium. These experiments will demonstrate where the distal helix peptide binds and if it occludes the LxVP binding site.

Proteolytic studies suggest that calcium binding in the N-terminal lobe of CnB causes it to bind the CnBBH and release the RD. Assuming the fluorescent studies above reveal distal helix sequence binding to CaN373, the release of the RD can be tested by measuring the anisotropy of the same peptide, testing its binding to CaN373 both in the absence and presence of calcium. Introduction of calcium into the system should cause the release of the peptide, decreasing its anisotropy. Also, measurement of the binding of the LxVP motif peptide to CaN373 via anisotropy measurements with/without calcium and the distal helix peptide will reveal if release of the distal helix exposes the LxVP motif. Additionally, we can use site-directed mutagenesis to disrupt the calcium binding sites in the N-terminal lobe of CnB. This disruption should prevent CnB N-terminal lobe binding to the CnBBH even under high-calcium conditions. Using this CnB mutant in CaN373, we can determine if disrupting calcium binding changes RD binding to the CnBBH and if disrupting calcium binding changes LxVP motif binding.

We see that, in *drosophila*, the region of the RD that contains the distal helix is simply missing, yet CaN signaling obviously still works in *drosophila*. Thus, we can make the distal helix deletion in human CaN, as it is in *drosophila*, and examine the affect that it
has on CaN inhibition and activity at both high and low levels. Anisotropy measurements with this deletion construct and the fluorescent LxVP motif peptide ± calcium would confirm whether or not the distal helix sequence blocks the LxVP docking site under low calcium conditions. Also, we can further investigate the impact of the distal helix on CaN activity by testing the activity of this distal helix deletion mutant with and without calcium/CaM.

If the distal helix region interacts with CnB/CnBBH under low calcium conditions, it would become disordered when it is released upon CnB N-terminal lobe calcium binding. This is suggested by previous proteolytic studies, CaN crystal structure, previous CD spectroscopy, and our own CD and anisotropy studies.\textsuperscript{52,56,71-73} However, these studies are done in vitro and the solutions used are very dilute compared to the crowded conditions of the cell. It is possible that the crowding of a cellular environment causes a partial ordering of the RD that still allows CaM binding. As a proxy for crowding, the propensity for structure of the RD in the absence of CaM can be examined by CD spectroscopy and fluorescence anisotropy experiments with the RD in increasing concentrations of osmolytes such as trimethylamine N-oxide (TMAO) and 2,2,2-trifluorethanol (TFE). TFE is known to induce α-helix in peptide chains with a propensity for helix whereas TMAO can induce general structure. The low net charge per residue of the RD suggests that, while it is disordered, it is relatively compact and globular in shape. If the RD’s conformational ensemble is restricted in this way, addition of chaotropes, or denaturants, such as guanidine, urea, and thiourea could disrupt the compact, globular ensemble, affecting the CD spectra and anisotropies of the RD. Both the osmolytes and denaturants can be used as tools to further investigate the disordered state of the RD.

Finally, after an increase in calcium causes the N-terminal lobe of CnB to bind calcium and then bind to the CnBBH, releasing the RD; CaM is then able to bind the RD, induce formation of the distal helix, and removal of the AID from the catalytic site, activating CaN. The distal helix is amphipathic which necessitates a hydrophobic surface for its hydrophobic face to interact with. This surface is likely on the surface of CaM, allowing
the distal helix to undergo its hairpin-like chain reversal that would remove the AID from
the active site. To better understand this interaction, we must learn where on CaM the
distal helix binds. We can attempt to grow crystals of CaM with the RDC, as this is the
smallest piece of the RD that still includes the CaMBR and distal helix. If diffraction
quality crystals are obtained, we can use our CaM:pCaN crystal structure for molecular
replacement to help solve the structure and determine the distal helix placement.
However, as we have observed through anisotropy measurements and CD melts, the
distal helix is only partially stable. This may make it mobile in crystal form and its
placement on CaM unobtainable. If this is the case, we could employ NMR spectroscopy
to determine the structure of the RDe:CaM complex. The complex at 27.1kDa is a
tractable size for NMR.

As we have seen CaM binds to a disordered RD with 1:1 stoichiometry and causes a
compaction of the RD that includes the formation of the distal helix, an element
important for efficient CaN activity. This points to further investigation of the RD of CaN
when calcium levels are low to determine if the distal helix region occludes the LxVP
binding site; when calcium levels are increased but there is no CaM to observe the nature
of the disorder in the RD with osmolytes or denaturants; and when both calcium and
CaM are present to determine where on CaM the hydrophobic face of the distal helix
interacts. Our studies and literature thus far have led us to this model: at resting cellular
calcium levels the N-terminal lobe is devoid of calcium and not bound to the CnBBH.
This allows the distal helix region of the RD to interact with CnB/CnBBH, occluding the
LxVP substrate docking site. When calcium levels increase, the RD is released from its
interaction with CnB/CnBBH by the N-terminal lobe of CnB binding calcium and then
the CnBBH. Release of the RD from CnB/CnBBH exposes the LxVP site, allowing for
substrate docking at this site. Calcium-loaded CaM then binds the now disordered RD,
causing formation of the distal helix which folds upon CaM in a hairpin-like chain
reversal. The formation and chain reversal of the distal helix removes the AID from
CaN’s catalytic site, causing the full activation of CaN (Figure 6.2). Our work on
calmodulin activation of calcineurin has lead us to a better understanding of the
molecular mechanism by which this activation occurs. It also leads us to new questions, the study of which will give us a better understanding of how CaN substrate recognition is regulated. As our understanding of CaN regulation increases we are better positioned to investigate and control CaN in human health, especially with respect to the use of immunosuppressant drugs for organ and tissue transplant patients.
Figure 6.1: Sequence alignment of the RD

Sequence alignment of the RD from *S. cerevisiae*, *S. pombe*, *D. melanogaster*, and *H. sapien* is shown. The distal helix sequence is underlined in human. Of note is the insertion in *S. cerevisiae* and *S. pombe* in the region N-terminal to the CaM binding domain and the deletion of the distal helix in all non-human species shown.
Figure 6.2: Model for the activation of CaN by calcium/CaM

From left to right: at low calcium levels, the CnB N-terminal lobe is unbound by calcium and does not bind the CnBBH, allowing the RD to interact with this region. An increase in calcium causes the CnB N-terminal lobe to bind calcium and then the CnBBH, releasing the regulatory domain. The disordered domain is now available for calcium-loaded CaM to bind. CaM binding induces formation of a distal helix that allows for removal of the AID from CaN’s catalytic site, activating the enzyme.
References


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AWARDS AND HONORS

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PUBLICATIONS

Manuscripts Submitted

Dunlap, TB; Rumi-Masante; Lester, TE; Creamer, TP (2013). The distal helix in the regulatory domain of calcineurin is important for domain stability and enzyme function. Submitted.

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**RESEARCH PRESENTATIONS**

*Denotes Presenter

1. **Dunlap, TB***; Rumi-Masante, J; Rusinga, FI; Lester, TE; Holbrook, E; Zhang, X; DeRouchey, JE; Dunker, AK; Weis, DD; Creamer, TP (2012). Disordered regulation of calcineurin. *26th Annual Gibbs Conference on Biothermodynamics (Carbondale, IL)*

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