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Structural Studies of Calcium/Calmodulin Dependent Protein Kinase II Activation

By

Luke H. Chao

A dissertation in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Molecular and Cell Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in Charge:

Professor John Kuriyan, Chair
Professor Michael A. Marletta
Professor Kevan M. Shokat
Professor Ehud Isacoff

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Abstract

Structural Studies of Calcium/Calmodulin Dependent Protein Kinase II Activation
by
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Cell signaling utilizes the frequency of calcium stimuli to produce diverse physiological outcomes. From fertilization of oocytes and cardiac facilitation, to muscle contraction and action potential firing, periodic fluctuations in calcium levels play a key role in determining cell fate. During my thesis work, I studied a protein signaling complex that responds to the frequency of calcium stimuli: calcium/calmodulin dependent protein kinase II (CaMKII). The CaMKII holoenzyme is a dodecameric kinase assembly that activates and autophosphorylates in a manner dependent on the frequency of calcium stimuli. Its best-studied role is in the post-synaptic neuron, where it responds to calcium pulse frequencies to initiate changes important for Long Term Potentiation.

My work investigated the activation mechanism of CaMKII, with the goal of understanding the enzyme's cooperative response to calcium-saturated calmodulin. This work elucidates the mechanism for CaMKII activation; by demonstrating that CaMKII is not a simple 'coincidence detector' as previously postulated, by showing that CaMKII is cooperatively activated by calcium-saturated calmodulin, and by demonstrating that cooperative activation is mediated by the inter-subunit 'capture' of regulatory segments. This work demonstrates that the cooperative response of CaMKII can by modulated by the length of the linker region where splice-form deletions and insertions occur. Similar modifications have been shown to shift the frequency-dependent activation of the enzyme, suggesting that our proposed mechanism enables the enzyme to tune its frequency response depending on developmental or tissue-specific expression. In addition, analysis of interactions exhibited by a peptide inhibitor of CaMKII revealed docking sites which other proteins, such as the NMDA-receptor, may utilize in order to localize and affect the frequency response of the CaMKII. Finally, I investigated the exchange of CaMKII subunits between holoenzyme complexes, and discuss the implications of such a process in prolonging the response to calcium stimuli beyond the lifespan of individual protein subunits.

For my family.

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Acknowledgments

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My intellectual development is the direct result of those in the lab who have taken the time and care to mentor and guide me. These individuals include all of the lab members I have had the pleasure to work with. Specifically, I wish to acknowledge Matthew Young, who was my introduction to the lab, and Jonathan Winger, who has been a source of guidance and support throughout my studies. I also wish to thank Nick Levinson and Sebastian Deindl both have been a constant source of advice and discussion.

I wish to acknowledge Oren Rosenberg and André Hoelz, whose previous work on CaMKII was a foundation upon which my thesis work is based, and Angus Nairn, whom provided advice and guidance at various stages of the project.

Finally, my work presented here would not have be possible without Patricia Pellicena's efforts and I am grateful for having the opportunity of working with her. I also wish to acknowledge Howard Schulman, with whom it has been a pleasure to discuss CaMKII biochemistry.

None of this work would have been possible without all of the support in the laboratory. Specifically, I wish to give my great thanks to Steven Jacques. I still do not know how he does it, but I feel extremely lucky to have been able to work in a lab which runs so smoothly. In addition, I wish to thank Xiaoxian Cao and Tiffany Chou for all of their support.

Special assistance from David King for peptide synthesis and mass spectrometry was vital for this project. And the fantastic support at the ALS beamlines, run by Corrie Ralston and her staff made this work possible.

Chapter 1:

Introduction

1.1 Overview

Calcium is an ubiquitous second messenger involved in regulating many cell signaling processes, including motility, exocytosis, apoptosis and excitability (Berridge et al., 2000). In calcium signaling, changes in the frequency of the stimulus produce diverse physiological outcomes (Boulware and Marchant, 2008).

Calcium signaling utilizing the frequency of the stimulus has advantages over simply amplitude-dependent signaling. For example, frequency dependent calcium signaling prevents inadvertent activation of other targets. This specificity is important in ensuring high fidelity signal transduction. There are many challenges in cellular calcium signaling. For example, the absolute change in cellular calcium concentration upon stimulation is relatively modest (from ~100s of nM to 1 μM (Sabatini et al., 2002)), requiring signaling proteins to respond to small changes in calcium concentration. Furthermore, the enzymes activated by calcium pulses have catalytic rates that are slow relative to the calcium pulse frequency, necessitating a means for the signaling protein to integrate many calcium pulses in order to produce an output. Finally, many of these enzymes are regulated by a multilayered series of inhibitory interactions, thereby setting the threshold for activation at a high level. To overcome these challenges, signaling proteins must respond to calcium pulses with great sensitivity in the time scale relevant to the calcium stimulus frequency.

The calcium/calmodulin dependent protein kinase II (CaMKII) holoenzyme is a dodecameric assembly which responds to both the amplitude and the frequency of calcium stimuli (De Koninck and Schulman, 1998). The calcium frequency range at which CaMKII responds is from 1 to 10 Hz (De Koninck and Schulman, 1998). Faithful transduction of calcium signals within this range by CaMKII is necessary because slight changes in calcium stimulus frequencies can result in dramatically different cellular changes (Mulkey et al., 1994). Molecular understanding of a mechanism for frequency-dependent response of CaMKII requires understanding the conformational changes that tie calcium concentration changes to enzyme autophosphorylation.

Calmodulin, an universal transducer of calcium signals, binds four calcium ions to undergo a conformational change which enables interaction with many targets, including CaMKII (Crivici and Ikura, 1995). The CaMKII assembly comprises twelve subunits, each composed of a kinase domain, a regulatory segment, and an association domain. The kinase domain contains the active site responsible for catalyzing phosphotransfer, the regulatory segment controls the catalytic activity of the kinase domain, and the association domain is necessary and sufficient for assembling the dodecamer in its oligomeric form (Hudmon and Schulman, 2002). The binding of calmodulin to the regulatory segment directly activates CaMKII. This enables phosphorylation of downstream substrates and trans-subunit autophosphorylation within a holoenzyme assembly. *In vitro*, it has been demonstrated that autophosphorlyation of the purified CaMKII is dependent on frequency of calcium stimuli (De Koninck and Schulman, 1998). The conformational changes that occur during activation result in autophosphorylation, and it is these changes that must be sensitive to the frequency of a calcium stimulus.

One mechanism that imparts signaling sensitivity on a protein is cooperativity in ligand binding. A cooperative response enables a molecular system to respond within the narrow ligand concentrations found in cells, ensuring low activity at low levels of stimuli and facilitating a sharp increase in activity at specific ligand thresholds (Goldbeter and Koshland, 1982). Binding of calcium-saturated calmodulin to CaMKII was shown to be a cooperative process (Gaertner et al., 2004). This observation suggests the presence of interactions that propagate through the CaMKII holoenzyme assembly upon activation. A common mechanism for cooperativity is the transmission of allosteric changes within a multidomain assembly, where a change in an individual subunit influences the ability of an adjacent subunit to undergo the same change. We sought to understand the conformational changes that occur during CaMKII activation, with the ultimate goal of understanding the mechanism of cooperativity in CaMKII activation.

During my thesis studies, I used biochemical and structural studies to understand the molecular steps that occur upon CaMKII activation by calcium/calmodulin. Specifically, I developed a structural understanding of the cooperative response of the enzyme to calcium/calmodulin by dissecting the interactions that occur between kinase domain subunits. This mechanism implies that once activation of an individual kinase subunit occurs, activation spreads to adjacent subunits in a sequential manner. This invalidates previous models of activation, and predicts that the enzyme's autophosphorylation process occurs efficiently, with few non-productive activation events that do not result in autophosphorylation. Future work will focus on direct measurements of the role of cooperativity in frequency detection by CaMKII to test the predictions made by this activation mechanism.

1.2 Calcium Signaling

The classical non-covalent regulatory mechanism is the binding by the divalent cation calcium (Clapham, 2007). As a second messenger, the signaling effects of calcium are commonly transduced through intermediary calcium binding proteins. These proteins often contain specific calcium-binding motifs or domains. The EF hand is a canonical calcium binding motif named after the 5th and 6th helices of parvalbumin (Kretsinger and Nockolds, 1973). In an EF hand, a loop residing between two orthogonal alpha helices cradles the Ca^{2+} ion using the carboxyl groups of an aspartate and glutamate residue, a backbone carboxyl group, and a coordinated water molecule. The calcium affinities for EF hands vary 100,000-fold, depending on loop affinity and protein core packing (Lewit-Bentley and Rety, 2000). Calmodulin, a ubiquitous calcium-binding signal transduction protein, contains four EF hands. Two EF hands reside in a N-terminal lobe, and two EF hands are in a C-terminal lobe. When four calcium ions bind to calmodulin, the two lobes of calmodulin rearrange, exposing a ridge of hydrophobic residues in each lobe. Exposure of these residues enables the two lobes of calmodulin to wrap around a target motif, often an alpha-helical portion of an enzyme (Figure 1.1 (Crivici and Ikura, 1995)). The fundamental importance of calmodulin is illustrated by the fact that different calmodulin expression levels resulted in the diversity of finch species observed by Charles Darwin during his voyage aboard the Beagle (Abzhanov et al., 2006).

Calmodulin-recognition motifs are variable and reflect degenerate modes with which calmodulin may recognize its target. Calmodulin targets include phosphatases, kinases, nucleotide exchange factors and other signaling proteins. Often, calmodulin binding releases an inhibitory interaction, thereby transducing calcium binding into a conformation change in the target enzyme. The conformational change caused by calmodulin then regulates the enzyme's catalytic activity. In protein kinases, substrate binding interfaces are often sterically occluded by sequences recognized by calmodulin, and the calmodulin binding region has an additional function as a pseudosubstrate (Kemp and Pearson, 1991). In an elaborate calmodulin/target interaction, the anthrax edema factor adenylate cyclase is recognized by calmodulin with one lobe bound to calcium and another in the *apo* state (Figure 1.1 E) (Drum et al., 2002).

CaMKII can be activated by sub-saturated forms of calmodulin, (Shifman et al., 2006). The activation of CaMKII by calcium is a highly cooperative process, with a Hill coefficient of 4.4 (Bradshaw et al., 2003). This level of cooperativity reflects the cooperative binding of calcium to calmodulin (Hill coefficient of ~1.5), and the cooperative binding of calmodulin to CaMKII (Hill coefficient of ~3). The structural basis for cooperative calcium binding to calmodulin has been well characterized (Crivici and Ikura, 1995). In this work, I investigated the conformation changes that occur upon binding of calcium-saturated calmodulin to CaMKII.

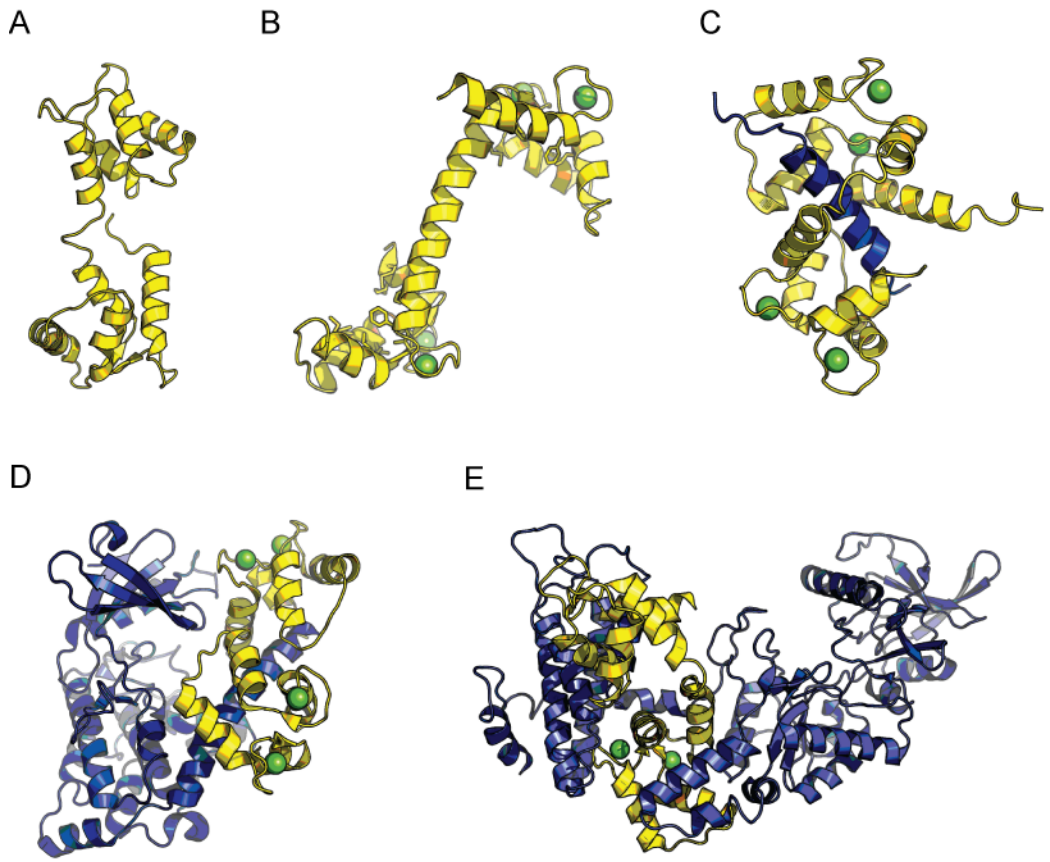


Figure 1.1: Target recognition by calcium/calmodulin is diverse.

The calcium-induced conformational changes in calmodulin and the recognition by calmodulin of target enzymes. **A.** Calmodulin has a bilobal structure. There are two EF hands in each lobe. In its *apo*, calcium-free form, the four EF hands of calmodulin (shown as yellow in cartoon representation) form a relaxed conformation (PDB code: 1CFD, (Kuboniwa et al., 1995)). **B.** Calcium ions (shown as green spheres) bind to each of the EF hands (PDB code: 1CLL, (Chattopadhyaya et al., 1992)), exposing hydrophobic residues (shown in yellow sticks representation). **C.** Calcium-bound calmodulin typically binds to alpha-helical portions of their targets. Shown is calcium-saturated calmodulin bound to the target helix of myosin light chain kinase (shown in blue in cartoon representation) (PDB code: 2BBM, (Ikura et al., 1992)). **D.** Calmodulin may utilize additional non-hydrophobic interactions in target recognition. For example, calmodulin utilizes additional hydrogen bonding interactions with the N-terminal lobe of the death-associated protein kianse (DAPK) (PDB code: 2X0G (de Diego et al., 2010)). **E.** Sub-saturated calmodulin with two calcium ions bound activates the anthrax edema factor adenylate cyclase by dislodging an autoinhibitory block from the active site. One lobe of calmodulin recognizes the target in the calcium-free form (PDB code 1K93, (Drum et al., 2002)).

1.3 Frequency-dependent calcium signaling

The frequency of cellular calcium stimuli spans a 10^7–fold range to regulate physiological processes that occur on timescales ranging from hours to milliseconds (Figure 1.2) (Boulware and Marchant, 2008). Circadian rhythms result from 24-hour calcium oscillations (Ikeda et al., 2003). The fertilization of an oocyte by sperm is dependent on calcium oscillations observable by light microscopy (Gilland et al., 1999). In cardiac tissue, the fluctuation of calcium concentration regulates muscle contraction (Haddock and Hill, 2002). In the nervous system, the frequency of calcium-regulated action potential firing is a central form of information, and recorded calcium pulse frequencies vary depending on the region of the brain (Koester and Sakmann, 2000; Spitzer, 2006). The exocytosis of synaptic vesicles occurs at the timescale of microseconds (Berridge Nat Rev. Cell Biol), and is dependent on fast calcium frequencies at the presynaptic terminal. Thus, the ability of signaling proteins to respond to calcium stimuli in a frequency-dependent manner underlies the diversity observed in calcium signaling outputs.

Changes in intracellular calcium levels are regulated through the complex interplay of calcium channel gating, calcium concentration buffering and calcium efflux systems (Berridge et al., 2003). Extracellular calcium concentrations are typically at mM concentrations. A variety of active processes are required to maintain resting cytosolic concentrations at levels ~100 nM. Changes in intracellular calcium are a consequence of influx from extracellular sources and the release of calcium from intracellular compartments. Upon activation by an external stimulus, ion channels open and rapid calcium influx occurs. Binding of external ligands or changes in membrane potential can gate and modify the properties of calcium channels. Repeated, rapid calcium pulses require influx and efflux systems that quickly shape calcium concentration changes. Downstream proteins then transduce these dynamic changes in calcium level into transcriptional programs in the cell.

Specific activation of particular downstream signaling proteins is critical, and thus must be under tight regulatory control. For example, activation of CaMKII is mediated by calmodulin, but calmodulin also has many additional targets. In neuronal signaling, calmodulin also activates a phosphatase, calcineurin. Inadvertent activation of calcineurin is undesirable, as it results in an opposing neuronal signaling process, and thus it is necessary for cells to have a way to differently activate signaling proteins using the same calmodulin activator.

One means to achieve specificity in calcium signaling is through distinct frequency-dependent activation of calcium signaling proteins. Frequency is a robust means for signal transduction. Consider two proteins whose activation is dependent on the amplitude of the calcium concentration. When the specific activation of protein A over protein B is critical, any accidental variation in calcium concentration could inadvertently activate protein B. However, if the two proteins have different responses dependent on the *frequency* of calcium stimuli, any slight changes in calcium concentration will have a less dramatic effect on the signaling output. If calcium pulse frequency is the signal to which the protein responds, calcium concentration variation would thus be less likely to lead to inadvertent activation of other pathways.

CaMKII activation is sensitive to both the amplitude and frequency of a calcium stimulus. This may be especially critical in the physiological context of a neuron, where calcium stimulus

frequency is an important parameter in signaling. Different calcium frequencies sets the threshold between opposing synaptic fates: long-term depression (LTD) and long-term potentiation (LTP) (Bear, 1995). The paradigm for the LTD/LTP threshold is that long term depression results from low frequency (less than 1 Hz) activation of protein phosphatase I and/or calcineurin, and long term potentiation is mediated by high frequency (greater than ~5 Hz) activation of protein kinases (Dudek and Bear, 1992; Mayford et al., 1995). This model posits that a frequency-specific response is responsible for signaling specificity. Frequency-dependent activation also requires the existence of a threshold for activation. Below this threshold frequency activation does not occur, and above the frequency threshold a cooperative enzyme would rapidly activate.

Specific activation of calcium signaling proteins also requires great sensitivity. The total calcium concentration change during cell signaling is less than 10-fold (~100 nM to 1 μM). In order for calcium to control signaling output, the responding proteins must respond within this narrow concentration range. One means to achieve such sensitivity is through a cooperative system. A Cooperativity protein can more rapidly increase activity as ligand concentration is increased, compared to a non-cooperative enzyme. Many multi-protein systems show frequency-dependent cooperative activation. For example, NF-kB and Ras have been shown to activate in a frequency-dependent manner in cells and in modeling studies (Dolmetsch et al., 1998; Kupzig et al., 2005). The Ras system demonstrates cooperative activation (Hill coefficient of ~5) (Yasuda et al., 2006). Understanding of the molecular basis for cooperativity requires an understanding of allosteric regulatory mechanisms. In the next section I discuss allostery and its role in propagating conformational changes important for a cooperative response.

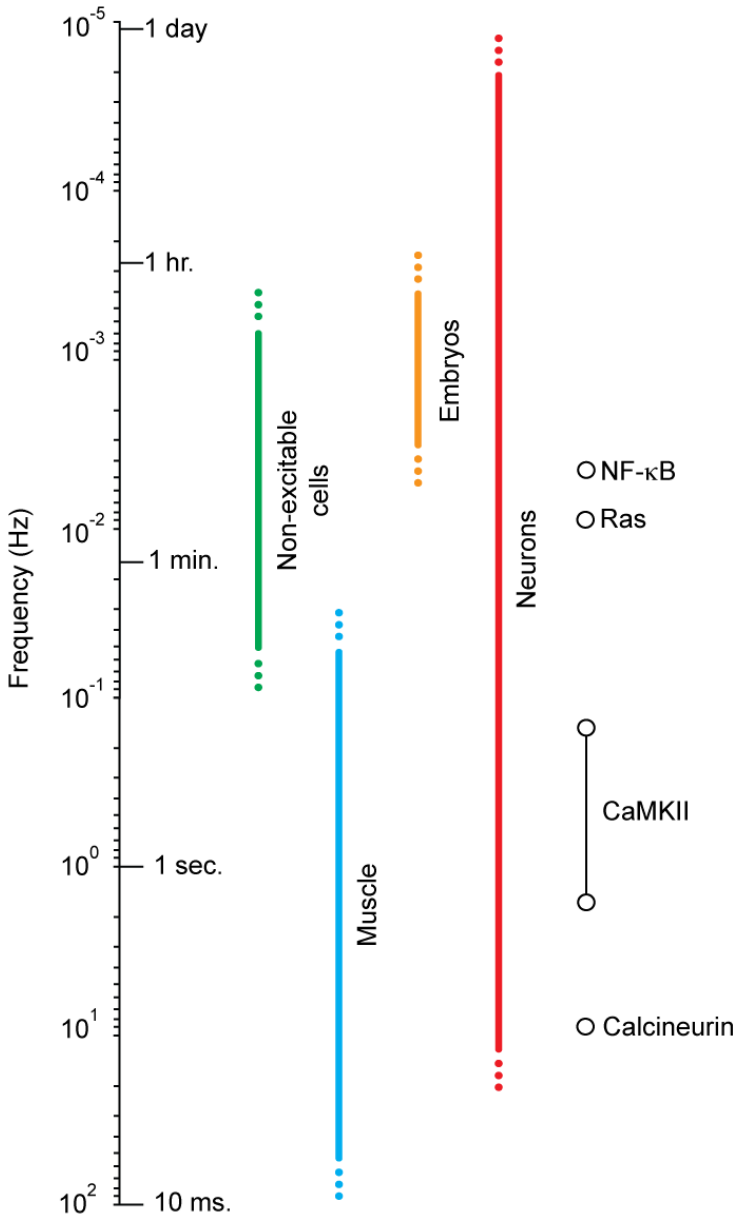


Figure 1.2: Physiological calcium frequencies span a wide range.

The range in frequency of calcium oscillations observed in different cell and tissue types is shown. (Adapted from Boulware and Marchant, 2008). Indicated in empty circles are frequency for half maximal activation of different proteins which respond in a frequency-dependent manner, including calcineurin (Colella et al., 2008), CaMKII (De Koninck and Schulman, 1998), the Ras Erk/Map kinase pathway (Kupzig et al., 2005), and the NF-kB pathway (Dolmetsch et al., 1998).

1.4 Allostery in the Cooperative Activation of Macromolecular Complexes

The binding of calcium-saturated calmodulin to CaMKII is cooperative. This implies that binding at a given subunit in the CaMKII holoenzyme influences subsequent calmodulin binding events at other subunits. A common means for such 'transmitted' conformational changes is allostery, whereby conformational changes influence the binding or activity of other subunits in an oligomer. Monod describes allostery as:

> "... indirect interactions between distinct binding sites (allosteric effects) ... these interactions are mediated by some kind of molecular transition (allosteric transition) which is induced or stabilized by the protein when it binds an allosteric ligand." (Monod et al., 1965)

In classical cooperative systems allosteric changes are transduced in a symmetric fashion within a symmetric oligomer, as observed for hemoglobin. Examples of multidomain allosteric systems are abundant in cell signaling. Allosteric changes in protein kinases are essential for their regulation, and allosteric regulation is a common means by which multiple events are integrated in autoinhibition. In the Src tyrosine kinase, the coupled binding of the SH2 domain to Tyr527 and the SH3 domain to the polyproline motif are both necessary for the proper down-regulation of the kinase (Sicheri et al., 1997; Xu et al., 1997; Young et al., 2001). In the regulation of the nucleotide exchange factor Vav1, multiple interactions stabilize autoinhibitory interactions between a regulatory segment and the catalytic site (Yu et al., 2010). The release of this interaction follows the sequential phosphorylation of residues in this region and disassembly of a multidomain assembly. In these systems, binding at a distant site influences activity.

When multiple active sites are under allosteric control, cooperative activation can occur. A molecular understanding of allostery can explain important phenomena such as the cooperative binding of a ligand, or activation of a signaling protein (Cui and Karplus, 2008). Tetrameric human hemoglobin is the prototypical cooperative system that provides the foundation for understanding allostery and cooperativity. In hemoglobin, a structural mechanism for cooperativity describes how salt bridges link the ligand binding state of each monomer to structural stability of two quaternatery states (Perutz, 1989). Hemoglobin orthologs display different higher-order oligomeric interfaces and utilize different allosteric mechanisms. This diversity illustrates the multiple evolutionary paths to cooperativity (Kuriyan and Eisenberg, 2007).

Other effectors in addition to oxygen can influence the structural transitions of hemoglobin. The alkaline Bohr effect and the effect of 2,3-bisphosphoglycerate on hemoglobin's physiological oxygen binding properties demonstrate that allosteric systems may have multiple effectors. In non-symmetric systems, where one active site is subject to multi-domain regulation, several allosteric changes are necessary for activation. With the case of CaMKII, we consider the possibility of multiple allosteric effectors influencing enzyme activation. In addition to calcium/calmodulin, I investigate inter-kinase domain interactions and the role they may play in allosteric interactions that might influence a cooperative response.

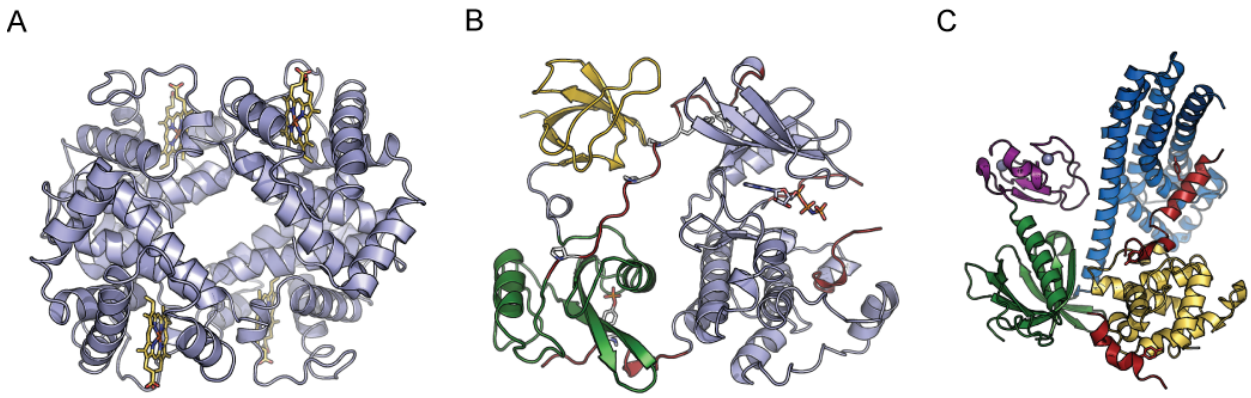


Figure 1.3: Multidomain allosteric systems

In allosteric systems, the conformation of one domain influences interactions at other sites. **A.** In tetrameric human hemoglobin, oxygen binding to a heme moiety results in changes at the interface between subunits that affect the oxygen affinities of hemes in adjacent subunits (PDB code 2HHB, (Fermi et al., 1984)). **B.** In Src kinases, the SH2 domain (show in cartoon in green) binding to Tyr527 and and SH3 domain (shown in cartoon representation in yellow) binding to the polyproline motif cooperate to hold the kinase domain in an autoinhibited state and these two sets of interactions are coupled (Young et al., 2001). **C.** In the Vav guanine nucleotide exchange factor, activation of the catalytic Dbl homology domain (DH, shown in blue cartoon representation) is mediated by release of an acidic domain (red) which requires the the disassembly of a network of interactions between the calponin homology (yellow) and pleckstrin homology (green) domains (Yu et al., 2010).

1.5 Physiological roles of CaMKII

CaMKII is the only know system where a frequency-dependent response to stimuli has been demonstrated *in vitro* (De Koninck and Schulman, 1998). Alternative mRNA splicing of CaMKII transcripts results in changes in the length of the linker region between the regulatory and association domains. These linker modifications can modulate the frequency dependent response of these enzyme (Bayer et al., 2002b). This observation suggests that the frequency-sensitive component of CaMKII activation is an interaction that can be strengthened or weakened, thereby shifting the frequency dependence for autophosphorylation. These properties are consistent with CaMKII's proposed function in setting the frequency threshold in post-synaptic neuronal signaling.

CaMKII's best characterized function is in responding to calcium signals in the post-synaptic neuron (Lisman et al., 2002). The landmark knock-in studies of the autophosphorylation site (Thr 286) demonstrated that mice containing a single mutation (Thr 286 to Ala) showed dramatic defects in behavioral memory tasks (Silva et al., 1992b). Subsequent studies characterized the importance of CaMKII in the initiation of Long Term Potentiation, the major experimental setting for studying the strengthening of the synaptic connections in learning and memory (Silva et al., 1992c). A prominent feature of electron micrographs of synapses is the dense post-synaptic density, where CaMKII comprises ~10% of the protein material (Sheng and Hoogenraad, 2007). The function of such high concentrations of enzyme at the post-synaptic density, while a topic of great speculation, remains unknown. While the frequency-dependent activation of CaMKII has been observed *in vitro*, it remains undemonstrated if this function sets the frequency response of each neuronal dendrite. Any potential *in vivo* verification of this hypothesis requires molecular understanding of the mechanism for frequency-specific activation.

1.6 CaMKII Structure

CaMKII is expressed in approximately 20 different variants due to alternative mRNA splicing (Tombes et al., 2003). All variants contain a basic domain architecture comprising a N-terminal kinase domain, a regulatory segment, and a C-terminal association domain. High-resolution crystal structures have been solved of the association and kinase domains in isolation (Hoelz et al., 2003; Rosenberg et al., 2005). Small-angle X-ray scattering reconstructions suggest that a compact array of radially arranged kinase domains is released upon calcium/calmodulin activation (Rosenberg et al., 2005).

The kinase domain is comprised of a bilobal structure, with a mainly β-sheet N-terminal portion (N-lobe), and a predominately alpha-helical C-terminal portion (C-lobe) (Knighton et al., 1991). The kinase domain is held in the autoinhibited state by interactions made by the regulatory segment with the C-lobe of the kinase domain. These interactions distort helix αD, which may affect the kinase domain's affinity for ATP. A prominent feature of the autoinhibited kinase domain is an antiparallel coiled-coil comprised from the regulatory segment, which mediates formation of an autoinhibited dimeric unit of kinases (Rosenberg et al., 2005).

The association domain forms the central hub around which the kinase domain units are arranged. Each domain is wedge-shaped and comprised of single α-helix and a curved anti-parallel β-sheet. Each of the protomers is assembled into six-membered rings in the holoenzyme assembly. In the holoenzyme, two hexameric rings are stacked with the beta-sheet surface mediating this interface. The association domain α-helix is the N-terminal connection point to which the kinase domain regulatory segment connects.

Between the kinase domain and regulatory segment is an intervening variable region, which is the site of splicing-specific insertions and deletions which produce the more than 20 different expressed forms of CaMKII subunits (Tombes et al., 2003). Structures of the full-length protein are not available, so it is unknown if the association domains undergo any conformational change which differs between the full-length protein and isolated domain. Furthermore, knowledge of the specific arrangement of kinase domains relative the association domains requires high resolution structures of the full-length CaMKII holoenzyme.

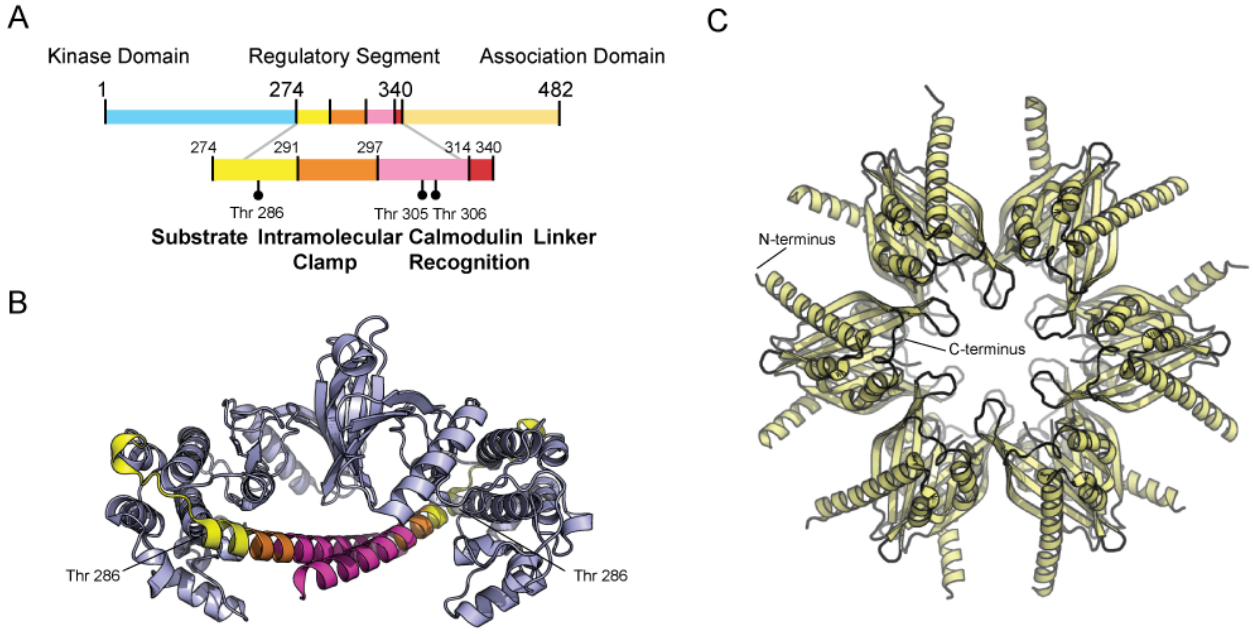


Figure 1.4: CaMKII structures

A. Each subunit of the CaMKII holoenzyme comprises a kinase domain, a regulatory segment and a C-terminal association domain. Within the regulatory segment are three phosphorylation sites, which control CaMKII activity. **B.** The crystal structure of the isolated kinase domain revealed an autoinhibited dimer mediated by an antiparallel coiled-coil formed by the regulatory segments (PDB code 2BDW, (Rosenberg et al., 2005)). **C.** The association domain forms a dodecameric hub assembly around which kinase domains are arranged (PDB code 2UX0).

1.7 Outstanding questions in CaMKII regulation

The CaMKII holoenzyme responds to the frequency of calcium stimuli to propagate downstream changes essential for Long Term Potentiation in neurons and calcium channel activation in cardiac tissue (Anderson et al., 1994). A molecular mechanism for calcium frequency-dependent activation is necessary for understanding how the autophosphorylation results from specific calcium stimuli. Small-angle X-ray scattering studies suggest that the kinase domain assembly becomes less compact upon calcium/calmodulin binding (Figure 1.5). CaMKII has been demonstrated to bind calcium-saturated calmodulin cooperatively (Bradshaw et al., 2003). It is not know what the molecular basis for this cooperative binding is, and what role cooperativity may play in the enzyme's frequency dependent response.

In this work, I describe studies of a molecular system that is capable of responding to the frequency of calcium pulses. I first attempted to understand cooperative activation of CaMKII by calcium-saturated calmodulin (Chapter 2). Patricia Pellicena, a former postdoctoral fellow in the Kuriyan laboratory, initiated these studies, and components of her work as well as the work of co-authors are included here to aid in the presentation of a coherent story. It is with pleasure that I acknowledge this work was done with several individuals who provided critical components for the development of this work's conclusions. Howard Schulman's expertise in CaMKII biochemistry was instrumental in shaping our experiments and for developing our understanding of this mechanism. Sebastian Deindl contributed a stochastic simulator and solved analytical solutions for the allosteric systems. Lauren Barclay contributed important efforts in the co-crystallization of the kinase domain and peptide inhibitor CaMKIINtide. These components are noted in the text. The work presented in chapter 2 has been published elsewhere, and permission to use this previously published work has been obtained from all co-authors.

The next steps in this work are to develop understanding of CaMKII's frequency-dependent activation mechanism. We have initiated experiments to test the role of the cooperative activation mechanism in the frequency response of CaMKII utilizing a pulsed perfusion device, with the goal of performing single-molecule measurements. As this work is ongoing, it will not be discussed here. Another component of CaMKII regulation I have investigated is the exchange of subunits between CaMKII holoenzyme and its consequences in the maintenance of an activating CaMKII signal. This work is presented in the final chapter (Chapter 3).

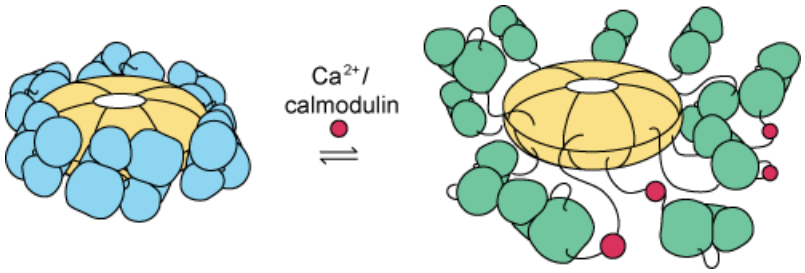


Figure 1.5 CaMKII holoenzyme activation

Crystallography, electron microscopy and small angle X-ray scattering experiments have led to a general activation model of CaMKII. In these models, the holoenzyme is present as a compact arrangement of kinase domains prior to calcium calmodulin binding. Activation is proposed to release kinase domains from this compact autoinhibited state, and to free kinases domains to autophosphorylate one another. Prior to this work, it was unknown what interactions mediate this process.

Chapter 2:

Intersubunit capture of regulatory segments is a component of cooperative CaMKII activation

2.1 Overview

Calcium-calmodulin dependent protein kinase II (CaMKII) is unique among the protein kinases because it is known to respond not just to the strength of the activation signal but also to its frequency (De Koninck and Schulman, 1998). This property, which is manifested in the ability of CaMKII to escape calcium-dependence at high calcium spike frequency, is likely to underlie the essential role for CaMKII in the strengthening of synaptic connections between neurons by Long Term Potentiation (Lisman et al., 2002), and in the response to and control of heart rate (Anderson et al., 1994; Lisman et al., 2002). The frequency response of CaMKII relies on the Ca^{2+}/calmodulin-dependent phosphorylation of a specific threonine residue (Thr286 in the mouse α isoform) in one kinase domain by another once a critical spike frequency is crossed, resulting in calcium-independent activity (autonomy) (De Koninck and Schulman, 1998). Mutation of Thr286 in CaMKII has dramatic effects, most notably the impairment of spatial learning in mice that bear this mutation (Giese et al., 1998; Silva et al., 1992a).

Another unique feature of CaMKII is its assembly into large symmetrical holoenzymes in which twelve kinase domains are tightly packed around a central ring-shaped scaffold (Gaertner et al., 2004; Hoelz et al., 2003; Rosenberg et al., 2005). This raises a conceptual challenge in understanding how the enzyme is regulated. Since trans-phosphorylation of Thr286 is a key step in the activation process, it is difficult to understand how uncontrolled phosphorylation is prevented in such a holoenzyme assembly, which concentrates kinase domains to an extraordinary extent. One model for frequency decoding posits that CaMKII is a "coincidence detector", in which the activation of kinase domains within a holoenzyme ring occurs stochastically as calcium levels rise (Hudmon and Schulman, 2002). Since adjacent kinase domains need to bind Ca^{2+}/calmodulin in order to switch on (Hanson et al., 1994; Rich and Schulman, 1998), the time required for this to happen would be linked to the onset of transphosphorylation and the consequent acquisition of calcium independence (see Figure 2.1 A) (Fong et al., 1989; Miller and Kennedy, 1986; Miller et al., 1988; Waldmann et al., 1990).

The simplest form of a coincidence detection model, illustrated in Figure 1a, is based on the fact that isolated and autoinhibited CaMKII kinase domains, separated from the holoenzyme assembly, bind to Ca^{2+}/calmodulin in a 1:1 stoichiometry and are activated without cooperativity. It has also been observed, in studies using a particular synthetic peptide substrate known as autocamtide, that the activation of the holoenzyme by Ca^{2+}/calmodulin occurs without apparent cooperativity (Bradshaw et al., 2003). This result was interpreted to mean that Ca^{2+}/calmodulin binding to one kinase subunit within a holoenzyme is independent of binding to other subunits. The uncorrelated binding of Ca^{2+}/calmodulin to the holoenzyme is the essential feature of this model that introduces the time delay before transphosphorylation can occur (Figure 2.1 A).

Despite the elegance of this model, other findings concerning CaMKII make it difficult to understand how such a coincidence detection mechanism might actually work. The binding of calcium-saturated calmodulin to the CaMKII holoenzyme shows clear evidence for positive cooperativity (Gaertner et al., 2004; Rosenberg et al., 2005). This suggests that the binding of one molecule of Ca^{2+}/calmodulin to a kinase subunit, which is expected to activate it, also potentiates adjacent kinase subunits for binding to Ca^{2+}/calmodulin (Figure 2.1 B). A crystal structure of the autoinhibited kinase domain of CaMKII revealed that the autoinhibitory segment, which contains the Ca^{2+}/calmodulin binding site, forms an antiparallel coiled coil within dimeric kinase domains (Rosenberg et al., 2005). The formation of autoinhibited dimers within the

holoenzyme would explain why Ca^{2+}/calmodulin binds cooperatively, because the first binding event would necessarily disrupt the coiled coil formed by the autoinhibitory segment and thereby increase the binding affinity of the holoenzyme for a second Ca^{2+}/calmodulin (Figure 2.1 B). These studies did not analyze the cooperativity of kinase activation by Ca^{2+}/calmodulin, but because kinase activation results from Ca^{2+}/calmodulin binding, it follows that activation should also be cooperative with respect to Ca^{2+}/calmodulin. The acquisition of autonomy (i.e., the phosphorylation of Thr286 following activation) is indeed cooperative with respect to Ca^{2+}/calmodulin (De Koninck and Schulman, 1998), which is also difficult to understand in terms of the simplest coincidence detector model.

In this paper we reexamine the cooperativity of CaMKII activation by Ca^{2+}/calmodulin by measuring the activity of the holoenzyme for two peptide substrates. One of these is autocamtide, which was used in the previous studies and is based on the sequence of a portion of the regulatory segment of CaMKII (Hanson et al., 1989). The other, referred to as syntide, is based on the sequence of a CaMKII phosphorylation site in glycogen synthase (Woodgett et al., 1983). Our measurements using these substrates show that both the enzyme from *Caenorhabditis elegans* and the mammalian γ isoform are activated cooperatively by Ca^{2+}/calmodulin. The Hill coefficients that we obtain from these measurements are as high as 4.3, depending on the construct used, suggesting that the binding of the first Ca^{2+}/calmodulin molecule to one kinase subunit potentiates several other kinase subunits for Ca^{2+}/calmodulin binding (Figure 2.1 C). Thus, binding and activation follow the same pattern, and the CaMKII subunits do not behave independently. Based on these results, CaMKII is unlikely to function as a simple coincidence detector.

Why did the earlier reports conclude that activation of CaMKII by autocamtide was not cooperative? We find that that although both autocamtide and syntide show cooperative activation, the observed degree of cooperativity is lower for autocamtide. Since this peptide is based on the regulatory segment of CaMKII, we turned to an analysis of the role that this segment might play in the cooperativity of the activation process. We have determined a new crystal structure of a truncated form of CaMKII in which the regulatory segment of one kinase (the substrate, bearing the critical Thr286 residue) is bound at the active site of the other (the enzyme). This structure shows that the regulatory segment from the substrate interacts extensively with the kinase domain of the enzyme. This raises the possibility that Ca^{2+}/calmodulin-bound subunits with open active sites can capture the regulatory segments of adjacent autoinhibited kinase domains, thereby causing the activation to "spread" by potentiating the sequential binding of additional Ca^{2+}/calmodulin, in a manner extending beyond dimeric units (Figure 2.1 D). We present biochemical evidence that supports a role for such a substrate-capture mechanism in increasing the cooperativity of Ca^{2+}/calmodulin binding to CaMKII. The reduction of apparent cooperativity that is seen with autocamtide might arise from interference with this mechanism, and the cooperativity that is actually present might simply have been missed in previous analysis.

How does the enzyme prevent runaway transphosphorylation upon calcium stimulation, which would short circuit frequency detection? By showing that the simple coincidence detection model cannot be operative, our work emphasizes the need for future experiments aimed at explaining how the activity of the kinase domains is controlled in a way that sets the timing for transphosphorylation appropriately.

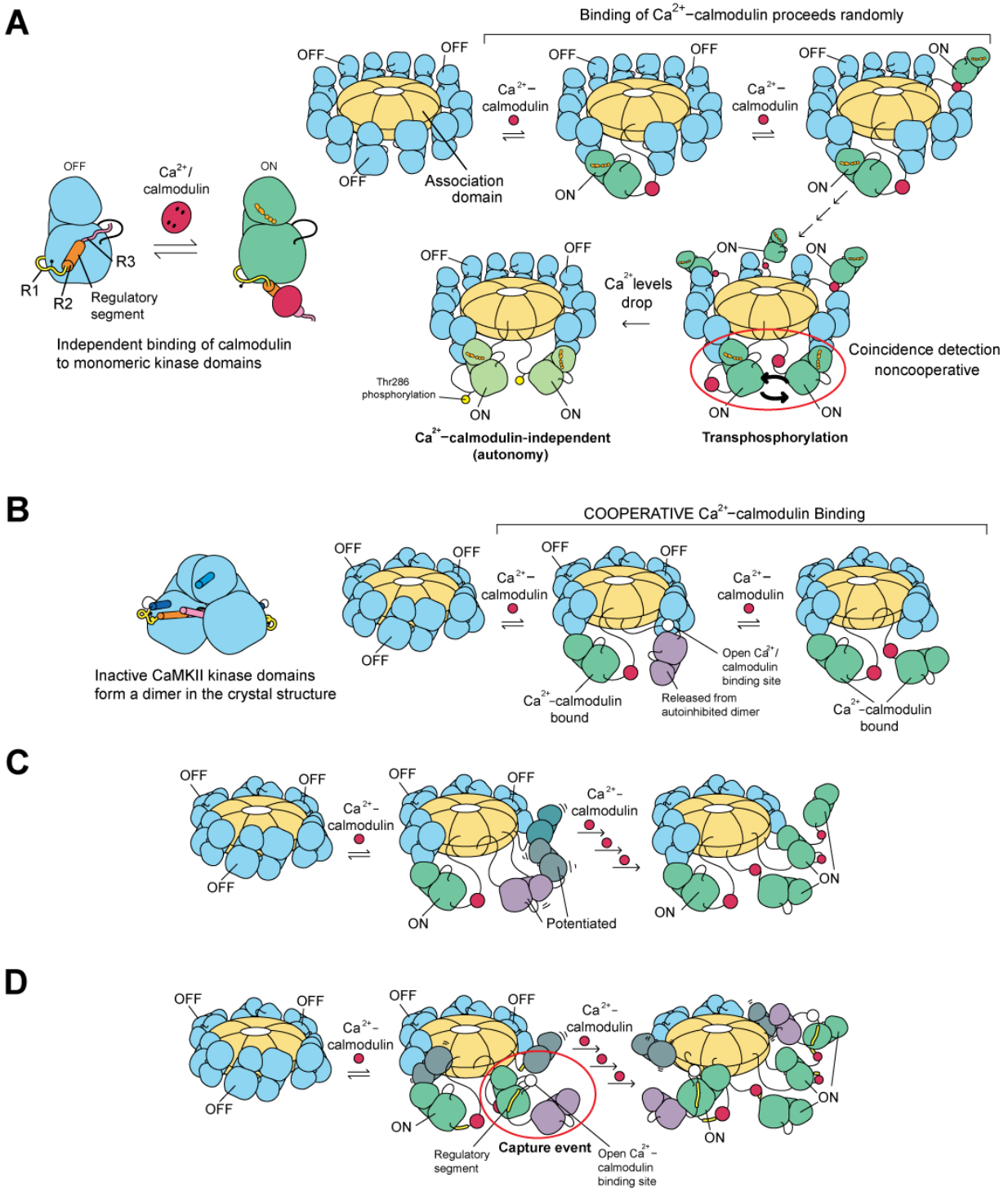

A
Binding of Ca2+–calmodulin proceeds randomly
OFF
ON
Ca2+–calmodulin
Association domain
Ca2+/ calmodulin
R1
R2
R3
Regulatory segment
Independent binding of calmodulin to monomeric kinase domains
Ca2+ levels drop
Thr286 phosphorylation
Ca2+–calmodulin-independent (autonomy)
Transphosphorylation
Coincidence detection noncooperative
B
COOPERATIVE Ca2+–calmodulin Binding
Inactive CaMKII kinase domains form a dimer in the crystal structure
Open Ca2+/ calmodulin binding site
Ca2+–calmodulin bound
Released from autoinhibited dimer
C
Potentiated
D
Capture event
Regulatory segment
Open Ca2+–calmodulin binding site

Figure 2.1 Cartoon schematic of models for CaMKII activation.

A. In a coincidence detection model, Ca^{2+}/calmodulin binding and activation occurs stochastically. Activation without cooperativity allows for CaMKII transphosphorylation to occur only in response to coincident and adjacent Ca^{2+}/calmodulin binding events. It is not known if autophosphorylation is bidirectional for nearest-neighbor subunits. **B.** Binding of Ca^{2+}/calmodulin is cooperative (Gaertner et al., 2004; Rosenberg et al., 2005), and autoinhibited kinase domains form a dimer in the crystal (Rosenberg et al., 2005). In a holoenzyme comprising autoinhibited dimers, Ca^{2+}/calmodulin binding to one kinase in the dimer would release the Ca^{2+}/calmodulin binding site of a second kinase domain. **C.** This work demonstrates that activation of CaMKII is cooperative, thus CaMKII is not a simple coincidence detector. The level of cooperativity observed indicates sequential activation of kinase domains beyond dimers, where activation of a kinase subunit potentiates activation beyond a second subunit (shown as grey-blue subunits). **D.** The capture of regulatory segments as substrates for adjacent kinase domains increases the cooperativity of activation. Only the activation and potentiation through subunit capture are shown, with any subsequent autophosphorylation omitted for simplicity.

2.2 Results and discussion

2.2.1 Activation of CaMKII by Ca^{2+}/calmodulin is cooperative when exogenous peptide substrate is used

The essential component of CaMKII activation is displacement by Ca^{2+}/calmodulin of a regulatory segment that otherwise blocks the active site (Hudmon and Schulman, 2002). The regulatory segment follows the kinase domain, and contains three elements, denoted R1, R2 and R3 (Figure 2.2 A). Unlike many protein kinases, the catalytic activity of CaMKII does not depend on phosphorylation of the activation loop, located near the active site (Johnson and Lewis, 2001). Instead, the regulatory segment blocks the active site in the absence of Ca^{2+}/calmodulin and sequesters the R1 element, bearing Thr286, in a channel adjacent to helix αD of the catalytic domain (Figure 2.2 A) (Rosenberg et al., 2005). The sequence motif recognized by calmodulin begins seven residues downstream of Thr286, and extends through the R3 element. The R2 and R3 elements form an α helix, with the R2 element clamping the regulatory segment to the kinase domain.

Phosphorylation of Thr286 requires two Ca^{2+}/calmodulin binding events: one resulting in activation of the subunit that serves as the enzyme and the other releasing Thr286 in the substrate subunit (Hanson et al., 1994; Rich and Schulman, 1998). The crystal structure of the autoinhibited CaMKII kinase domain shows a dimer in which the R2 and R3 elements of the regulatory segment form an intermolecular, antiparallel coiled coil (Rosenberg et al., 2005). The C-terminal lobes (C-lobes) of the two kinase domains in the dimer are at either end of the ~40 Å long coiled coil, which serves to keep the Thr286 residue in one kinase domain far away from the active site of the other (Figure 2.2 A). Isolated kinase domains without the association domain are monomeric in solution, even when the regulatory segment is present. The crystallographic dimer may only be formed in the holoenzyme, where kinase domains are at high local concentration.

Calmodulin binding releases the regulatory segment from the kinase domain, thereby exposing Thr286 and making it available for phosphorylation. The rebinding of the regulatory segment to the catalytic domain is prevented by Thr286 phosphorylation, even in the absence of Ca^{2+}/calmodulin (Colbran et al., 1989). The R3 element also contains two autophosphorylation sites, Thr305 and Thr306, which prevent rebinding of Ca^{2+}/calmodulin when phosphorylated.

We measured the activity of full-length wild type *C. elegans* CaMKII holoenzyme (residues 1-482) towards two different peptide substrates as a function of Ca^{2+}/calmodulin concentration, using a continuous spectrophotometric assay to measure reaction progress (see Methods). One of these peptides, known as autocamtide, was used in the earlier work and its sequence (KKALRRQE<u>T</u>VDAL, with phosphorylation site underlined) is derived from the Thr286 autophosphorylation site of CaMKII (Hanson et al., 1989). The other peptide, known as syntide (PLARTL<u>S</u>VAGLPGKK, with phosphorylation site underlined), is derived from glycogen synthase, an exogenous CaMKII substrate (Woodgett et al., 1983). We use saturating levels of Ca^{2+} (200 μM), so that the measurements report on the cooperativity of Ca^{2+}/calmodulin binding to CaMKII, rather than that of Ca^{2+} binding to calmodulin. Although the activation of CaMKII under sub-saturating calcium concentrations is likely to be relevant (Lucic et al., 2008; Shifman et al., 2006), it has been demonstrated that the frequency response of CaMKII is preserved under conditions where calmodulin is saturated with Ca^{2+} (De Koninck and Schulman,

1998). Standard Hill analysis was used to determine cooperativity (Figure 2.3), and the Hill coefficient was determined by a numerical fit to the reaction velocity as a function of Ca^{2+}/calmodulin concentration (see Methods).

The EC_{50} value for Ca^{2+}/calmodulin is 55 ± 1.0 nM when syntide is used as a substrate, with a Hill coefficient of 3.0 ± 0.3 (Figure 2.2 B). For a system with two coupled binding sites, positive cooperativity results in Hill coefficients greater than 1.0 but less than 2.0 (Figure 2.4). The observation that the Hill coefficient is greater than 2.0 indicates that three or more subunits of the holoenzyme are coupled in the activation process.

There are more than 20 different forms of mammalian CaMKII as a result of alternative splicing insertions and deletions in the linker region between the kinase and association domains (Tombes et al., 2003). Different isoforms are expressed in a tissue-specific and developmentally timed manner (Hudmon and Schulman, 2002), and have been shown to have different Ca^{2+} frequency responses to Ca^{2+}/calmodulin (Bayer et al., 2002a). To examine the effect of linker length, we made constructs of *C. elegans* CaMKII in which we shortened the linker between the regulatory segment and the association domain, either by deleting 17 residues or by deleting the linker completely. We also lengthened the linker by inserting six residues that are expected to be flexible (with the sequence SAGSAS) between residues 327 and 328, as indicated in Figure 2.2 C. The length of the linker has a marked effect on the Hill coefficient, with the shorter linkers yielding higher Hill coefficients (Figure 2.2 C). The maximum value of the Hill coefficient was obtained for the CaMKII Δ17 construct in which 17 residues are deleted (Hill coefficient of 4.3 ± 0.2 compared to 2.7 ± 1.0 for the wild type enzyme). Making the linker longer and more flexible decreases the Hill coefficient to 1.7 ± 0.2. The linker length varies in different isoforms of mammalian CaMKII, and our results suggest that this may reflect the tuning of the Ca^{2+} responsiveness between different isoforms.

We measured the activity of full-length mammalian CaMKII (*S. scrofa* γ isoform) towards syntide as a function of increasing Ca^{2+}/calmodulin concentration. The Hill coefficient is 2.0 ± 0.2, showing that cooperative activation for syntide is also observed in a mammalian isoform (Figure 2.5 A). The *S. scrofa* γ isoform linker region is 15 residues longer than the wild type *C. elegans* CaMKII, consistent with the observed inverse correlation between linker length and Hill coefficient (Figure 2.2 C). We also measured the activity of *C. elegans* CaMKII with all the major regulatory phosphorylation sites (Thr286, Thr305 and Thr306) mutated to alanine. The Hill coefficient for Ca^{2+}/calmodulin activation of this mutant form is 2.9 ± 0.6, indicating that cooperative activation is a process that does not require autophosphorylation (Figure 2.5 B).

Previous measurements of the activation of the mouse α isoform of the CaMKII holoenzyme by Ca^{2+}/calmodulin utilized an end-point radiometric assay and autocamtide as a substrate, and yielded an EC_{50} value of 48 ± 6 nM and a Hill coefficient of 1.1 ± 0.2 (Bradshaw et al., 2003). By repeating the syntide measurements with a radiometric assay we verified that our observation of positive cooperativity for syntide phosphorylation is not due to differences between the spectrophotometric and radiometric assays (Figure 2.5 C). We used the spectrophotometric assay to compare the degree of cooperativity in Ca^{2+}/calmodulin activation of the *C. elegans* Δ17 construct with syntide and autocamtide as substrates. The Hill coefficient derived from these measurements is 3.9 ± 0.2 for syntide and 1.8 ± 0.3 for autocamtide (Figure

2.2 D). Thus, our new data show that the activation of CaMKII by Ca^{2+}/calmodulin is cooperative for both substrates, although the apparent degree of cooperativity is somewhat lower with autocamtide.

To visualize the degree of cooperativity more directly, a graph of $\log_{10}\left(\frac{f}{1-f}\right)$ versus $\log_{10}$[CaM] where *f* is the fraction of maximal specific activity, is shown in Figure 2d for the activity of the *C. elegans* CaMKII Δ17 construct towards syntide. The Hill coefficient is the slope of $\log_{10}\left(\frac{f}{1-f}\right)$ versus $\log_{10}$[CaM], and the graph confirms that the Hill coefficient is ~3.0 for syntide as a substrate, and ~2 for autocamtide.

We also measured the activity of *C. elegans* CaMKII holoenzyme towards autocamtide and syntide at saturating concentrations of Ca^{2+}/calmodulin. The K_M values for CaMKII towards autocamtide and syntide are 6.4 ± 0.8 μM and 150.0 ± 24 μM, respectively (Supplemental Fig. 1d). These results show that the reduction in cooperativity observed for autocamtide is correlated with a lower K_M value for autocamtide as a substrate. Both sequences contain the core canonical Ser/Thr recognition motif: RXX(T/S)Φ (where X represents any amino acid, and Φ represents a hydrophobic amino acid), indicating that interactions outside this motif are responsible for a lower K_M value for autocamtide as a substrate. Autocamtide is an artificial substrate based on the regulatory segment of CaMKII (Hanson et al., 1989), and so we wondered whether the observed differences between autocamtide and syntide could reflect a role for a regulatory segment in cooperativity. As noted above, phosphorylation of Thr286 is not required for cooperative activation, and so the regulatory segment is not necessarily playing a role as a substrate in whatever process underlies cooperativity. The studies discussed below were aimed at dissecting the role of the regulatory segment in cooperativity.

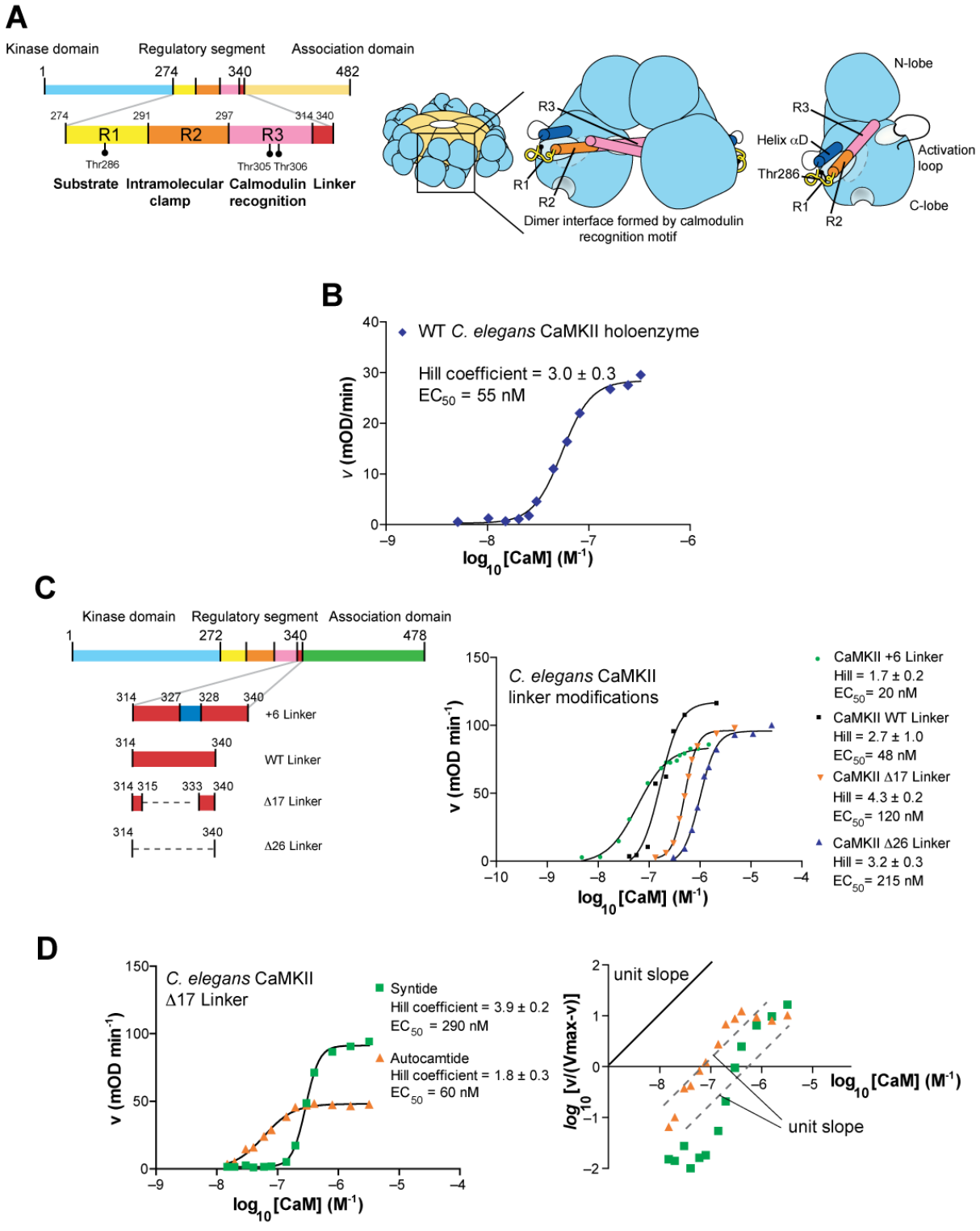

A
Kinase domain
Regulatory segment
Association domain
1
274
340
482
274
291
297
314 340
R1
R2
R3
Thr286
Thr305 Thr306
Substrate
Intramolecular clamp
Calmodulin recognition
Linker
R3
R1
R2
Dimer interface formed by calmodulin recognition motif
N-lobe
R3
Helix αD
Activation loop
Thr286
R1
R2
C-lobe
B
WT C. elegans CaMKII holoenzyme
Hill coefficient = 3.0 ± 0.3
EC50 = 55 nM
v (mOD/min)
log10[CaM] (M-1)
C
Kinase domain
Regulatory segment
Association domain
1
272
340
478
314
327
328
340
+6 Linker
314
340
WT Linker
314 315
333
340
Δ17 Linker
314
340
Δ26 Linker
C. elegans CaMKII linker modifications
v (mOD min-1)
log10[CaM] (M-1)
CaMKII +6 Linker
Hill = 1.7 ± 0.2
EC50= 20 nM
CaMKII WT Linker
Hill = 2.7 ± 1.0
EC50= 48 nM
CaMKII Δ17 Linker
Hill = 4.3 ± 0.2
EC50= 120 nM
CaMKII Δ26 Linker
Hill = 3.2 ± 0.3
EC50= 215 nM
D
C. elegans CaMKII Δ17 Linker
Syntide
Hill coefficient = 3.9 ± 0.2
EC50 = 290 nM
Autocamtide
Hill coefficient = 1.8 ± 0.3
EC50 = 60 nM
v (mOD min-1)
log10[CaM] (M-1)
unit slope
log10[v/(Vmax-v)]
log10[CaM] (M-1)
unit slope

Figure 2.2 Cooperativity of CaMKII activation by Ca^{2+}/calmodulin

A. Schematic diagrams of the CaMKII domain structure and regulatory segment arrangement in the autoinhibited state. Shown at left, the domain structure of CaMKII: the regulatory segment is enlarged, highlighting three elements: the R1 element, which contains the regulatory phosphorylation site Thr286; the R2 element, which clamps the regulatory segment to the kinase domain in the autoinhibited state; and the R3 element, which includes the calmodulin recognition motif. At right, a schematic diagram of the structure of the autoinhibited kinase domain: schematic of arrangement of autoinhibited dimers of the kinase domain in the CaMKII holoenzyme, with individual kinase domain enlarged. In the autoinhibited state, the R1 element is sequestered in a cleft below helix αD, and the R2 element positions helix αD to prevent substrate access to the active site. **B.** Cooperative activation of *C. elegans* CaMKII holoenzyme occurs with a Hill coefficient of 3.0 ± 0.3. *C. elegans* holoenzyme phosphorylation of the peptide syntide was measured as a function of calmodulin concentration. **C.** The Hill coefficient for *C. elegans* CaMKII activation, for constructs with various linker lengths. The velocity of substrate phosphorylation at varying calmodulin concentrations is shown. The linker modifications within residues 314-340 are indicated in the schematic shown at left. In the +6 linker six flexible residues were introduced to the middle of the linker region. In the Δ17 linker, 17 residues were deleted from the middle of the linker region. In the Δ26 linker all 26 residues were removed from the linker region. **D.** Cooperativity of calmodulin activation is reduced by autocamtide for the *C. elegans* CaMKII Δ17 deletion construct. Activity of CaMKII Δ17 was measured towards syntide and autocamtide, and plotted on a velocity (v) vs. $\log_{10}$[CaM] plot (right panel), and $\log_{10}$ [v/(V_{max}-v)] vs. $\log_{10}$ [CaM] plot (left panel). All error bars and ± terms expressed are s.e.m.

Figure 2.3 Standard Hill Analysis (Sebastian Deindl)

The fraction of CaMKII molecules bound to Ca2+/calmodulin and the concentration of Ca^{2+}/calmodulin are denoted *f* and [CaM], respectively. In the absence of cooperativity, $log_{10}\left(\frac{f}{1-f}\right)$ is expected to vary linearly with log_{10}[CaM], with a slope of 1.0 (see Methods). The value of f is difficult to derive from an exact treatment of the invidiual binding equiliria for systems where cooperativity arises from more than two binding sites. The standard Hill analysis, which is not physically correct but is commonly used, makes the simplifying assumption that binding occurs in an all nor none manner:

$$\mathrm{CaMKII} + n\mathrm{CaM} \leftrightharpoons \mathrm{CaMKII} \cdot \mathrm{CaM}_n$$

Here, n denotes the number of molecules involved in synergistic binding, and for such a reaction $log_{10}\left(\frac{f}{1-f}\right)$ also varies linearly with the concentration of Ca2+/calmopdulin, but with a slope of n, which is defined as the Hill coefficient

$$log_{10}\left(\frac{f}{1-f}\right) = n\ log_{10}\left(\frac{[\mathrm{CaM}]}{\mathrm{EC}_{50}}\right)$$

Here EC_{50} is the concentration of Ca^{2+}/calmodulin at half maximal activity.

Figure 2.4 Hill coefficient for a protein with two allosterically coupled binding sites (Sebastian Deindl)

Consider a protein, P, with two interacting binding sites for a ligand, L. The binding of the ligand to the protein can be described by two sequential binding equilibria with dissociation constants K_{D1} and K_{D2}:

$$P + L \underset{K_{D1}}{\rightleftharpoons} P{\cdot}L \qquad (1)$$

$$P{\cdot}L + L \underset{K_{D2}}{\rightleftharpoons} P{\cdot}L_2 \qquad (2)$$

The ration of bound and unbound protein is related to the two dissociation constants and the free ligand concentration through the following equation:

$$\frac{f}{1-f} = \frac{\frac{[L]}{K_{D1}} + \left(\frac{[L]}{K_{D2}}\right)\left(\frac{[L]}{K_{D1}}\right)}{1 + \frac{[L]}{K_{D1}}} \qquad (3)$$

The Hill coefficient is the slope of $log_{10}\left(\frac{f}{1-f}\right)$, i.e. the derivative of $log_{10}\left(\frac{f}{1-f}\right)$ with respect to $log_{10}[L]$, evaluated at ligand concentration when $f = 0.5$:

$$\text{Hill coefficient, } n_H = \left.\frac{d \log_{10}\left(\frac{f}{1-f}\right)}{d \log_{10}[L]}\right|_{f=0.5} \qquad (4)$$

Evaluating this derivative allows the Hill coefficient, n_H, to be computed directly from the ration of the two dissociation constants:

$$n_H = \frac{2}{1 + \sqrt{\frac{K_{D2}}{K_{D1}}}}$$

$$n_H \approx 2, \text{when } K_{D2} \ll K_{D1}$$

$$\text{and } n_H = 1, \text{when } K_{D2} = K_{D1}$$

Equation 5 demonstrates the well known fact that the Hill coefficient for a system with two binding sites and positive cooperativity (i.e., $K_{D2} < K_{D1}$) is between 1.0 and 2.0.

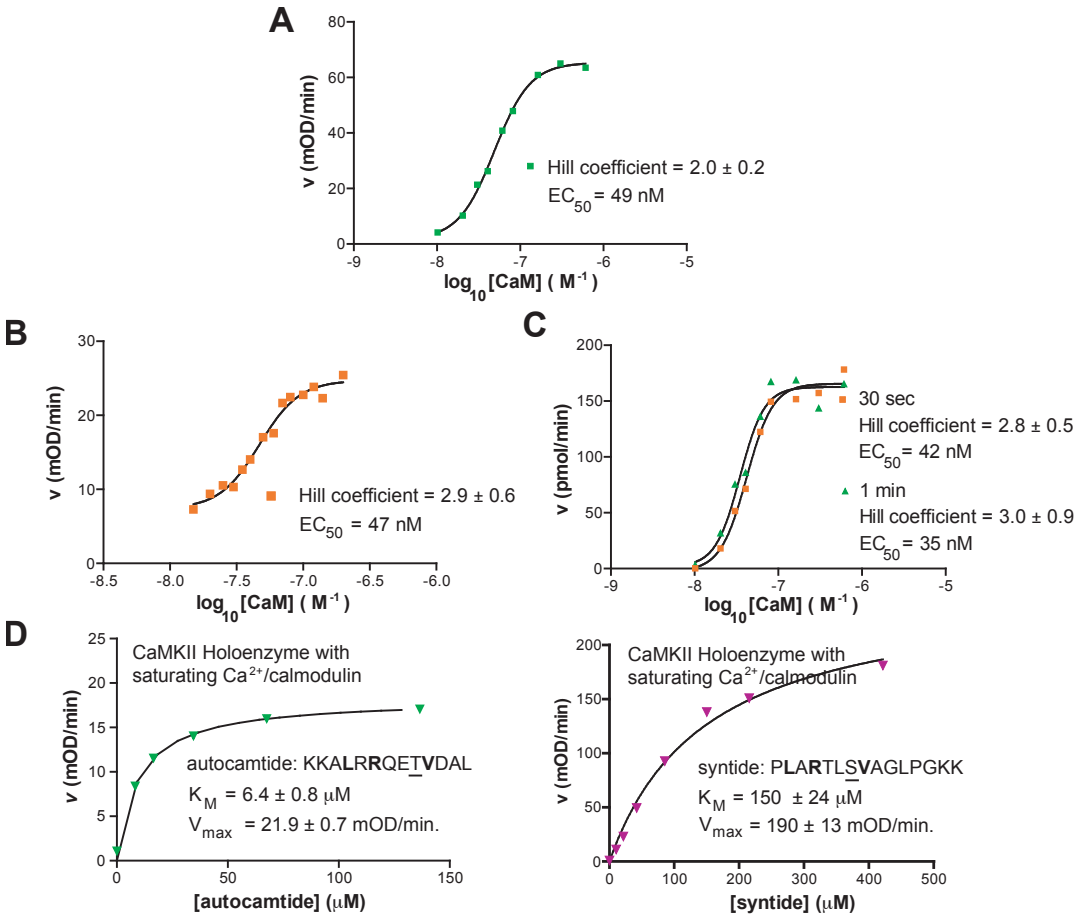


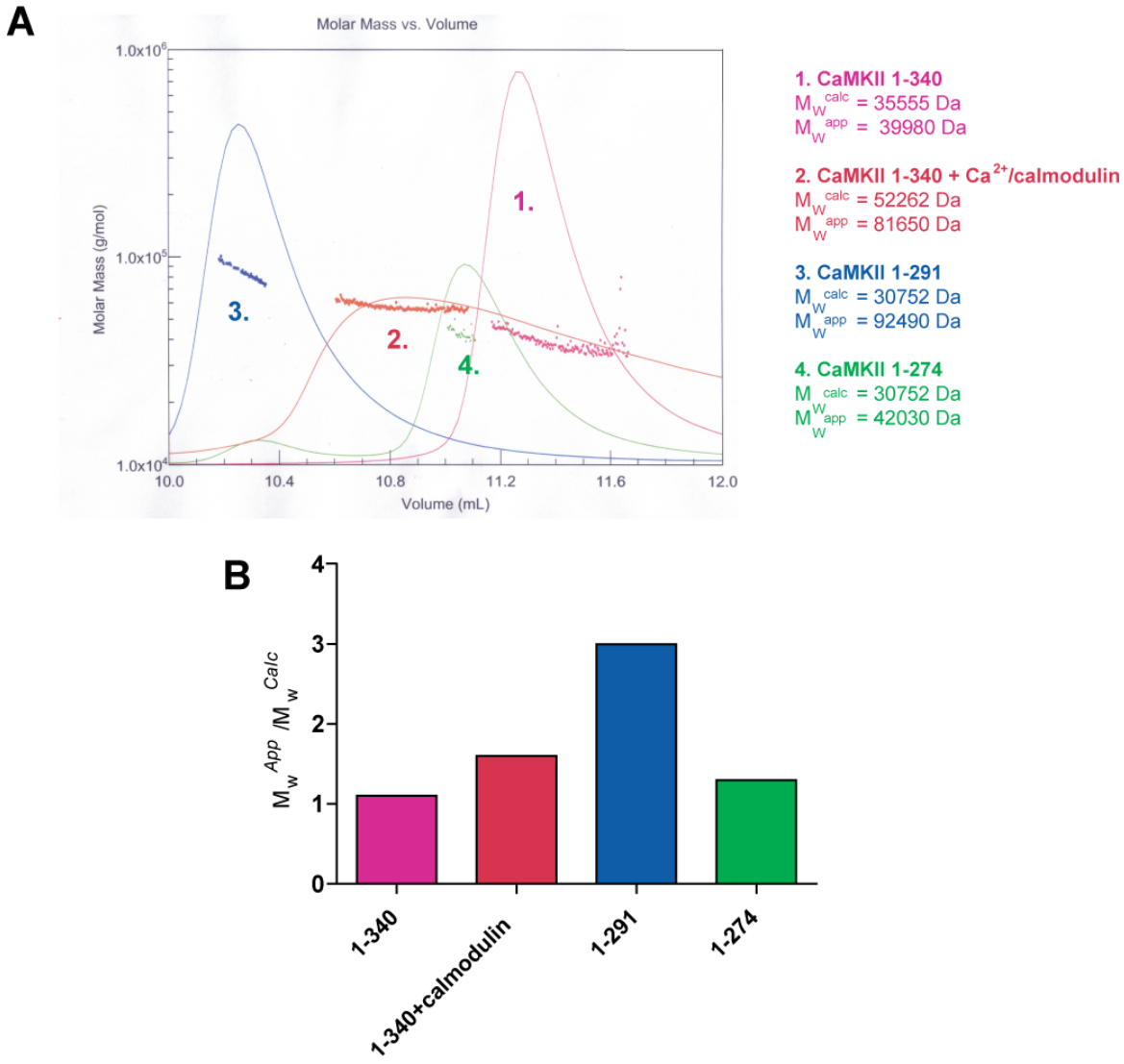
Figure 2.5 CaMKII activation

A. Mammalian CaMKII isoforms also demonstrate cooperative activation by Ca2+/calmodulin. The activity of the S. scrofa (pig) γ isoform of CaMKII was measured at varying calmodulin concentrations. The Hill coefficient is 2.0 ± 0.2. **B.** Cooperative activation by Ca^{2+}/calmodulin is independent of autophosphorylation. Mutation of all three regulatory phosphorylation sites (Thr 286, Thr 305 and Thr 306) to Alanine in the C. elegans CaMKII holoenzyme resulted in no change in the cooperative activation of CaMKII. **C.** Activation of *C. elegans* CaMKII holoenzyme by Ca^{2+}/calmodulin is cooperative in a radiometric assay of activity towards syntide. Activity of CaMKII towards syntide was measured using a radiometric filter binding assay which previously observed activity of CaMKII towards autocamtide was not cooperative. **D.** Activity of *C. elegans* CaMKII holoenzyme at saturating Ca^{2+}/calmodulin concentrations at varying concentrations of peptide substrate (autocamtide and syntide, left and right respectively). The sequences for the peptides are indicated in their respective panels with the canonical recognition residues indicated in bold, and the phosphorylated residue indicated by underline. The amounts of enzyme used in these measurements are 5 and 15nM respectively for autocamtide and syntide.

2.2.2 Light scattering measurements indicate that the R1 element mediates inter-subunit interactions upon calmodulin binding

A CaMKII construct containing the kinase domain and intact regulatory segment (but lacking the association domain) is monomeric in solution at concentrations below 100 μM, as observed previously (Rosenberg et al., 2005). The addition of Ca^{2+}/calmodulin to this construct shifts the population towards a predominantly dimeric form, as determined by multi-angle light scattering coupled to gel filtration (Figure 2.6). When the C-terminal portions of the regulatory segment (the R2 and R3 elements) are removed, the shorter construct (kinase and R1 element) is a multimer. A construct containing only the kinase domain, with the R1 element also removed, is monomeric at 100 μM concentration.

Figure 2.6 The R1 element of the autoinhibitory segment mediates inter-kinase domain interactions



C. elegans CaMKII constructs at 100 µM concentration were loaded onto a KW-803 size exclusion column pre-equilibrated in 20 mM Tris-HCl, pH 8.3, 250 mM KCl at a flow rate of 0.4 ml/min. The protein eluted from the chromatography system was detected by a coupled 18-angle light scattering detector and refractive index detector with a data collection interval of 0.5 s (Wyatt Technology Corporation, Santa Barbara, CA). **A.** Data analysis was performed using the program ASTRA (Wyatt Technology Corporation) which yielded an apparent molecular weight (M_W^{app}) for the 1-340 construct of 39980 Da, a molecular weight for the 1-340 construct with Ca^{2+}/calmodulin of 81650 Da, a molecular weight for 1-291 construct of 92490 Da, and a molecular weight for 1-274 of 42030 Da. **B.** Show in the bar graph below are the ratios of apparent and calculated molecular weights (M_W^{app}/M_W^{calc}) plotted for the respective constructs.

2.2.3 Structure of an enzyme-substrate complex of CaMKII kinase domains

The light scattering measurements suggest that release of the R1 element from the autoinhibitory interaction by Ca^{2+}/calmodulin allows it to form an alternative intermolecular interaction. In order to define the nature of this interaction we crystallized a CaMKII construct containing the kinase domain and the R1 element of the regulatory segment, but lacking the R2 and R3 elements. This construct crystallized readily in multiple crystal forms, and the structures of two crystal forms were determined in the absence of nucleotide (Forms A and B, Figure 2.8). The structure in crystal form A was determined at 1.7 Å resolution, and refined to a conventional R-value of 17.2% (free R-value of 20.7%). The structure in crystal form B was refined at 3.2 Å (conventional and free R-values of 27.0% and 32.9%, respectively).

In both structures the R1 element of the regulatory segment of one kinase domain is presented for phosphorylation at the active site of another (Figure 2.7 A). This interaction is repeated in a chain throughout the crystal lattice, with the R1 element of one kinase (referred to as the "substrate-kinase") inserted into the active site of the next one (referred to as the "enzyme-kinase"). The *in trans* interaction of the regulatory segment with a second kinase domain is essentially the same in the two crystal forms, although the orientation between the enzyme-kinase and the substrate-kinase is different. Unambiguous features in electron density maps indicate that the Thr286 residue is phosphorylated, which presumably occurred during protein expression or purification.

All eighteen residues of the R1 element (residues 274-291) are visualized in electron density maps, allowing identification of the connection between this element and the main body of the kinase domain from which it emanates. Sixteen of these residues (Arg276 to Val291) make contacts with the C-lobe of the enzyme-kinase. Asparagine 273 forms a pivot between the substrate–kinase and the R1 element. This pivot is flexible, as indicated by comparison with the form B crystal structure.

While this manuscript was being prepared a structure of a complex between calmodulin and a human δ CaMKII kinase domain construct with all three regulatory elements present was deposited in the Protein Data Bank (PDB code 2WEL) by the Structural Genomics Consortium. Intriguingly, this structure reveals a very similar chain of "enzyme-substrate" interactions, although the segment connecting the R1 element to the kinase domain (residues 275-280) is apparently disordered. Calmodulin is bound to the R3 element, not present in our structure, and does not make significant contact with the kinase domain. Along with other structures determined by our group and the Structural Genomics Consortium, a consistent picture of the interactions made by the regulatory segment emerges. When the intact regulatory segment (R1, R2 and R3) is present, but without calmodulin, the regulatory segments form a dimeric coiled-coil(Rosenberg et al., 2005). When the calmodulin recognition element (R3) is deleted, but R1 and R2 are present, the R2 element enters and blocks the active site because a coiled-coil can no longer be formed (seen in PDB entries 2VN9, 2VZ6, 2V70 and 3BHH). If calmodulin is bound to the R3 element, or if the R2 and R3 elements are deleted, an activated form of the kinase is obtained (the structures discussed in this paper, and PDB entry 2WEL).

The kinase domain is in an active conformation in our new structures(Lowe et al., 1997; Zheng et al., 1993) (Figure 2.7 B). The last eight residues of the R1 element of the substrate-kinase (Arg283 to Leu291) bind to the active site of the enzyme-kinase as seen previously for other kinase-substrate substrate-complexes (Lowe et al., 1997). The last eight residues of the R1 element therefore form a canonical substrate-docking interaction, and we refer to the region of the kinase domain that engages this portion of the R1 element as docking site A (Figure 2.7 C).

There are two other interactions made by the R1 element on the enzyme-kinase. A hydrophobic pocket is located in the C-lobe of the kinase domain, between helix αD and the C-terminal end of the activation loop, and it cradles Ile280 from the R1 element (Figure 2.7 C). This site, referred to as docking site B, is occluded in the autoinhibited form of the kinase due to rotation of helix αD (Rosenberg et al., 2005).

A cluster of acidic residues is located at the very base of the kinase domain, near the C-terminal end of helix αD and alongside helix αG, and interacts with Arg274 and Arg276 of the R1 element. This region, referred to as docking site C, orients the R1 element as it leaves the substrate-kinase and enters the channel leading into the active site of the enzyme-kinase (Figure 3c). Autocamtide contains several basic residues that are absent in syntide, and we speculate that these residues interact with docking sites B and C and are responsible for a lower K_M value for autocamtide as a substrate and, anticipating the discussion that follows, a reduction in cooperativity. Basic residues N-terminal to the canonical recognition sequence are also found in high-affinity cellular CaMKII substrates such as the N-methyl-D-aspartate glutamate (NMDA) receptor (Bayer et al., 2001; Liu et al., 2009). Utilization of docking site C in a high affinity substrate interaction is consistent with the observation that autocamtide can compete with binding of the NMDA receptor tail, while syntide cannot (Strack et al., 2000).

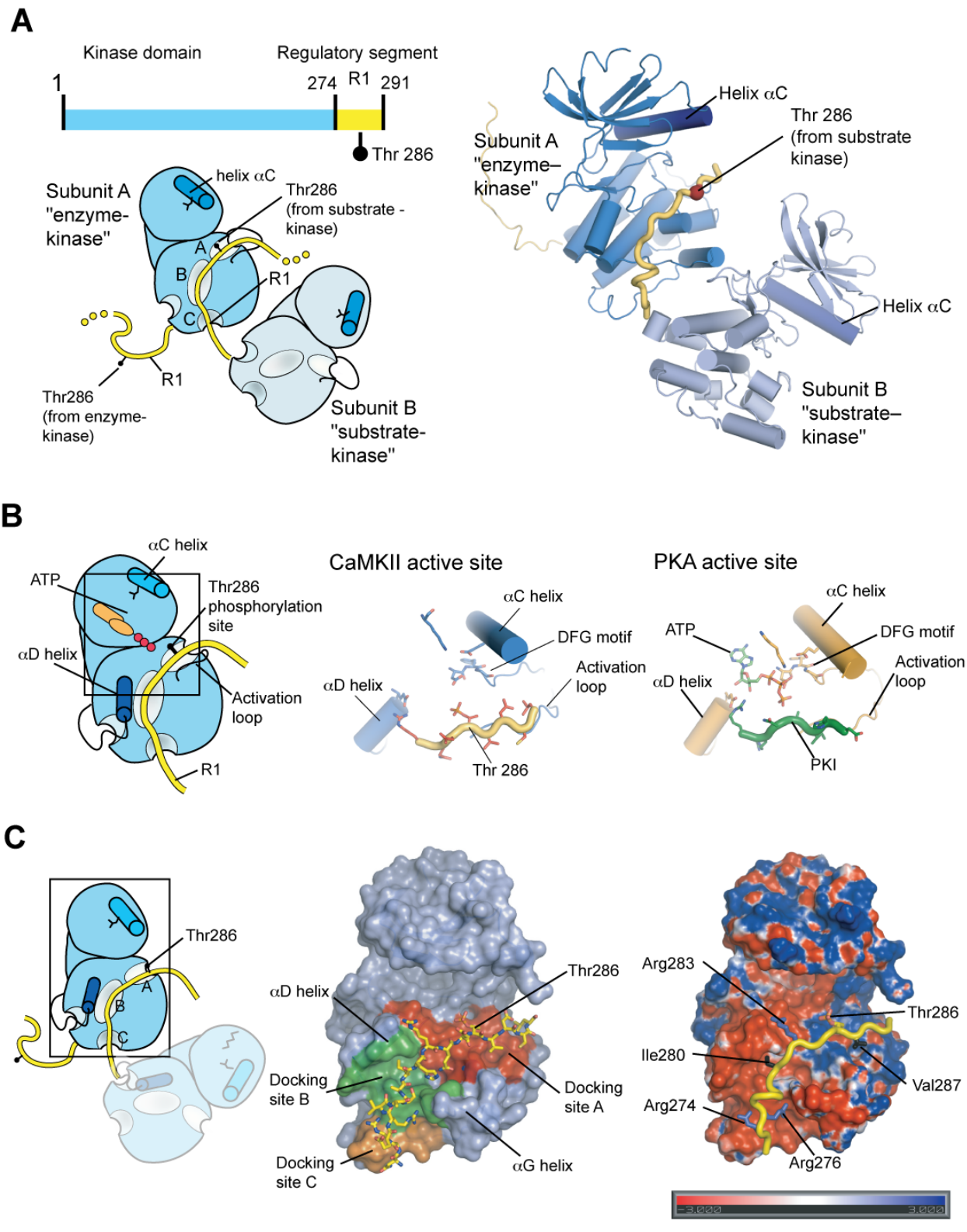

A
Kinase domain
Regulatory segment
1
274
R1
291
Thr 286
Subunit A
"enzyme-
kinase"
helix αC
Thr286
(from substrate -
kinase)
A
B
C
R1
R1
Thr286
(from enzyme-
kinase)
Subunit B
"substrate-
kinase"
Helix αC
Thr 286
(from substrate
kinase)
Subunit A
"enzyme–
kinase"
Helix αC
Subunit B
"substrate–
kinase"
B
αC helix
ATP
Thr286
phosphorylation
site
αD helix
Activation
loop
R1
CaMKII active site
αC helix
DFG motif
Activation
loop
αD helix
Thr 286
PKA active site
αC helix
ATP
DFG motif
Activation
loop
αD helix
PKI
C
Thr286
A
B
C
αD helix
Thr286
Docking
site B
Docking
site A
Docking
site C
αG helix
Arg283
Thr286
Ile280
Val287
Arg274
Arg276
-3.000
3.000

Figure 2.7 Crystal structure of the CaMKII enzyme-substrate complex

A. Schematic diagram of the crystallized CaMKII enzyme-substrate complex. The construct contains the kinase domain of CaMKII and the R1 portion of the regulatory segment. The structure shown (at right) is that of crystal form A. **B.** The CaMKII enzyme-substrate complex active site is in the active conformation. Comparison of the major active site components of the CaMKII enzyme-substrate complex with that of Protein Kinase A in the active state(Knighton et al., 1991). **C.** Docking sites utilized by the R1 element are indicated as docking sites A, B and C and colored red, green and gold, respectively on a surface representation of the kinase domain. The R1 element is shown in a sticks representation. At right, an electrostatic surface potential representation (produced with APBS (Adaptive Poisson-Boltzmann Solver) tools(Baker et al., 2001)) of the CaMKII kinase domain in the enzyme-substrate complex illustrates a negatively charged region (red) encompassing docking sites B and C that is utilized by residues in the R1 element (basic residues shown in blue, hydrophobic residues shown in black).

Figure 2.8 Table of data collection and refinement statistics

	Substrate Complex Form A 3KK8	Substrate Complex Form B 3KK9	CaMKIINtide Complex – 3KL8
Data collection			
Space group	P6522	P21	P21
Cell dimensions			
a, *b*, *c* (Å)	79.4, 79.4, 175. 5	37.7, 60.5, 70.2	72.4, 83.1, 145.1
a, *b*, *g* (°)	90, 90, 120	90, 93.9, 90	90, 101.9, 90
Resolution (Å)	68.7-1.7 (1.8-1.7) [#]	50.00-3.2 (3.3-3.2) [#]	83.1-3.4 (3.6-3.4) [#]
R_{sym} or R_{merge}	5.4 (59.6) *	12.7 (39.9)	16.1 (83.1)
I / sI	33.7 (2.8)	9.93 (2.3)	5.3 (1.2)
Completeness (%)	99.6 (94.9)	97.7 (87.7)	98.3 (99.9)
Redundancy	8.8 (6.1)	3.5 (3.1)	3.4 (3.3)
Refinement			
Resolution (Å)	68.7-1.7 (1.8-1.7)	50.00-3.2 (3.3-3.2)	83.1-3.4 (3.6-3.4)
No. reflections (unique/measured)	35411/578066	5171/8043	21523/282717
R_{work} / R_{free}	17.2%/20.7%	27.0%/32.9%	23.3%/28.5%
No. atoms			
Protein	4493	2204	10867
Ligand/ion	–	–	–
Water	258	–	80
B-factors			
Protein	37.34	91.22	75.71
Ligand/ion	–	–	–
Water	44.06	–	42.22
R.m.s. deviations			
Bond lengths (Å)	0.010 Å	0.004 Å	0.009 Å
Bond angles (°)	1.086°	0.798°	1.266°

[#] Data were collected from one crystal for each data set. *Values in parentheses are for highest-resolution shell.

2.2.4 Capture of regulatory segments results in cooperative activation

The extensive interactions between the R1 element of the substrate-kinase and the three docking sites on the enzyme-kinase suggests that the R1 element can play two distinct roles in the regulation of CaMKII. In addition to its role in sequestering Thr286 in the autoinhibited state, the R1 element in one subunit might serve as a molecular grappling hook that can be captured by an adjacent activated kinase domain. Such a capture would potentiate Ca^{2+}/calmodulin binding to the first subunit, increasing the cooperativity of the process.

To test this idea we measured the activity of isolated catalytic subunits, without the association domain. Note that the isolated kinase domain, even with the regulatory elements R1 to R3 present, is monomeric in solution (Kolb et al., 1998; Rosenberg et al., 2005; Shen and Meyer, 1998). Monomeric subunits are not capable of Thr286 trans-phosphorylation at the low concentrations (10 nM) we use in our assays.

Activity towards syntide substrate peptide was measured for the construct lacking the association domain, yielding an EC_{50} value of 803 nM with no cooperativity (Hill coefficient of 0.9 ± 0.1; Figure 2.9 A). Mass spectrometric analysis showed that although our preparations of holoenzyme are not phosphorylated, constructs of the kinase domain lacking the association domain are partially phosphorylated on Thr305 and Thr306 (data not shown). This is likely to account for the increased value of EC_{50} for activation by Ca^{2+}/calmodulin, which is ~10-fold higher than that observed for Ca^{2+}/calmodulin binding to a kinase-dead form of this construct (Rosenberg et al., 2005).

We added a 10-fold excess (100 nM) of a kinase domain inactivated by mutation (D135N, K42M), that lacks the regulatory segment. This "decoy" kinase domain has docking sites A, B and C unoccupied, and cannot bind to Ca^{2+}/calmodulin. The presence of the decoy lowers the EC_{50} values of Ca^{2+}/calmodulin to 380 nM, consistent with capture of the R1 element by the decoy and facilitation of Ca^{2+}/calmodulin binding to the autoinhibitor (Figure 2.9 B). Activation of the kinase domain shows apparent cooperativity with respect to the concentration of Ca^{2+}/calmodulin, with a Hill coefficient of 1.4 ± 0.1.

The non-hyperbolic response of the CaMKII kinase domain to Ca^{2+}/calmodulin in the presence of the decoy protein implies that the decoy protein and Ca^{2+}/calmodulin mutually facilitate the binding of each other to the enzyme. Binding of calmodulin to the enzyme releases its R1 element, which can then bind to the alternative docking sites on the decoy protein. In a reciprocal fashion, binding of the decoy protein to the R1 element releases the R2 and R3 elements for interaction with Ca^{2+}/calmodulin. We have modeled this system with a kinetic scheme, which predicts that the Hill coefficient for Ca^{2+}/calmodulin activation of the enzyme should be greater than 1.0, but less than 2.0 (Figure 2.10).

If the decoy subunit is mutated in docking site B to prevent binding of the R1 element (I205K, I101D, F98E, each mutation introduced separately), the activation by Ca^{2+}/calmodulin is no longer cooperative in the presence of the decoy (Figure 2.9 C). Mutation of residues in the R1 element that interact through docking site B (A280D, I281D, introduced together) in the full-length *C. elegans* holoenzyme likewise results in a reduction for the Hill coefficient for

Ca^{2+}/calmodulin dependent activation to 1.5 ± 0.1 (compared to a Hill coefficient of 3.0 ± 0.3 for the wild type holoenzyme), consistent with capture of the R1 element being one component of cooperative activation (Figure 2.9 D).

At the high local concentrations of the subunits within the holoenzyme, the R1 element could block the access of exogenous substrates by acting as a product inhibitor. This is consistent with the observation that the activity of Thr286 phosphorylated CaMKII in the absence of Ca2+/calmodulin is only 30%-70% that of fully activated Ca^{2+}/calmodulin bound CaMKII (Hanson et al., 1994).

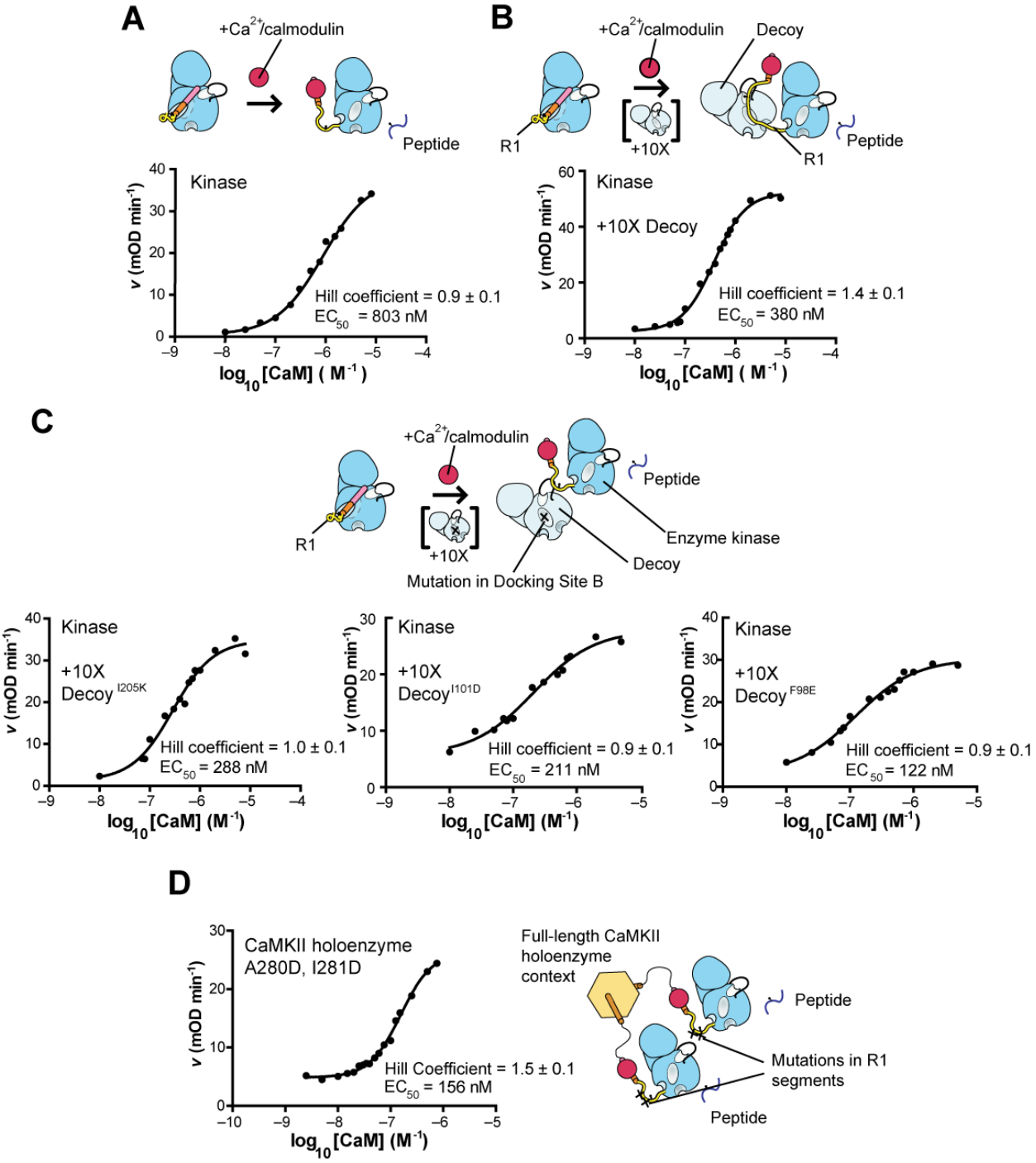
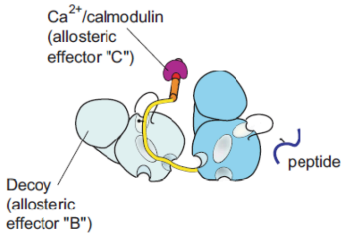

A
+Ca2+/calmodulin
Peptide
Kinase
v (mOD min-1)
Hill coefficient = 0.9 ± 0.1
EC50 = 803 nM
log10[CaM] (M-1)
B
+Ca2+/calmodulin
Decoy
R1
+10X
Peptide
Kinase
+10X Decoy
Hill coefficient = 1.4 ± 0.1
EC50 = 380 nM
log10[CaM] (M-1)
C
+Ca2+/calmodulin
Peptide
R1
+10X
Enzyme kinase
Decoy
Mutation in Docking Site B
Kinase
+10X
Decoy I205K
Hill coefficient = 1.0 ± 0.1
EC50 = 288 nM
log10[CaM] (M-1)
Kinase
+10X
Decoy I101D
Hill coefficient = 0.9 ± 0.1
EC50 = 211 nM
log10[CaM] (M-1)
Kinase
+10X
Decoy F98E
Hill coefficient = 0.9 ± 0.1
EC50 = 122 nM
log10[CaM] (M-1)
D
CaMKII holoenzyme
A280D, I281D
Hill Coefficient = 1.5 ± 0.1
EC50 = 156 nM
log10[CaM] (M-1)
Full-length CaMKII
holoenzyme
context
Peptide
Mutations in R1
segments
Peptide

Figure 2.9 Capture of the regulatory segment results in cooperative activation of a monomeric kinase domain

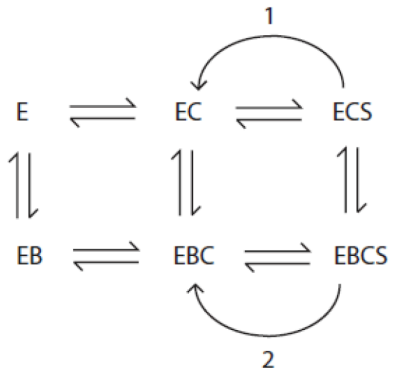
A. The activity of monomeric *C. elegans* CaMKII kinase domain towards syntide at varying calmodulin concentrations. The monomeric kinase domain does not exhibit cooperative activation (data from a representative Ca^{2+}/calmodulin activation response shown). **B.** Presence of a decoy kinase domain that is competent for capture of the R1 element results in cooperativity in the calmodulin activation of monomeric kinase domain. The decoy was added in 10X molar excess (100 nM), and has no enzyme activity because of mutations (D135N, K42M) introduced in the active site. **C.** Mutations in docking site B of the decoy eliminate cooperative activation of the monomeric kinase domain. Data for three mutations (I205K, I101D and F98E; each introduced separately) are shown. **D.** Mutation of residues (A280D, I281D, introduced together) in the R1 element in the *C. elegans* CaMKII holoenzyme holoenzyme results in a reduction in the Hill coefficient for Ca^{2+}/calmodulin activation. All error bars and ± terms expressed are s.e.m.

Figure 2.10 Kinetic scheme for a system with one active site influenced by two allosteric effector sites

We describe a model systen where the enzyme (E) is only capable of binding substrate (S) when Ca2+/camodulin (allosteric effector, “C”) is bound to the enzyme, and the binding of the decoy allosteric effector “B” facilitates binding of “C” and vice versa. For simplicity, we assume the fraction of enzyme that is bound by “B” but not by “C”, and that is capable of binding substrate, to be negligibly small.



The reaction mechanism of substrate conversion to product in the presence of the two effectors is modeled as follows:



Reactions 1 and 2, in the above scheme are the catalysis reactions

We applied a web-based implementation (www.biokin.com/king-altman/) of the King-Altman algorithm (King and Altman, 1956) to obtain a steady-state rate equation for product formation according to the above reaction scheme. This rate equation is a rational function of degree 2 in [C]. At fixed substrate concentration the rate equation is given by:

$$\frac{v([C])}{[E]_0} = \frac{a_2[C]^2 + a_1[C]}{b_2[C]^2 + b_1[C] + b_0}$$

where, v([C]) is the steady-state rate of product formation at a calmodulin concentration [C], $[E]_0$ is the total enzyme concentration, and the coefficients a_i and b_i are constants that depend only on the rate constants for the individual steps of the reaction scheme (not indicated in the scheme), at fixed substrate concentration.

$$\text{Since } v([C]) \rightarrow \frac{a_2}{b_2}[E]_0 = v_{max} \; for \; [C] \rightarrow \infty$$

$$v_{rel} = \frac{v([C])}{v_{max}} = v([C]) \cdot \frac{b_2}{[E]_0 a_2}$$

The equation for the relative product formation rate simplifies to:

$$v_{rel} = \frac{\alpha c^2 + \beta c}{\alpha c^2 + c + 1}$$

$$with \; \alpha = \frac{b_0 b_2}{{b_2}^2}, \beta = \frac{a_1 b_2}{a_2 b_1},$$

$$\text{and a rescaled calmodulin concentration, } c = \frac{b_1}{b_0}[C]$$

Analogous to discussion in Supplementary Discussion 1, a fraction *f* can be defined as

$$f = \frac{v_{rel}}{1 - v_{rel}} = \frac{\alpha c^2 + \beta c}{(1-\beta)c + 1}$$

The slope of a "Hill" plot is:

$$n_H(c) = \frac{d \ln f}{d \ln c} = \frac{c}{f}\frac{d f}{d c} = \frac{\alpha c}{\alpha c + \beta} + \frac{1}{(1-\beta)c + 1}$$

The Hill coefficient is maximal when:

$$c = \sqrt{\frac{\beta(1-\beta)}{\alpha}}$$

The maximum value of the Hill coefficient is given by:

$$n_{H,max} = \frac{2}{1 + \sqrt{\frac{\beta(1-\beta)}{\alpha}}} \leq 2$$

2.2.5 A feedback inhibitor of CaMKII binds to the kinase domain by mimicking interactions made by the R1 element

There is considerable similarity between the substrate-like binding mode of the R1 element of CaMKII and the docking of the protein kinase inhibitor PKI to protein kinase A (PKA) (Knighton et al., 1991). CaMKIINtide is a peptide inhibitor of CaMKII derived from the protein CaMKIIN, whose tissue-specific expression correlates tightly with that of CaMKII(Chang et al., 1998). A peptide inhibitor corresponding to residues 281-309 of the regulatory segment inhibits the activity of the isolated CaMKII kinase domain towards syntide with a K_i value of 0.2 μM (Colbran et al., 1989). We also measured CaMKIINtide inhibition of CaMKII kinase domain activity towards syntide, and obtained an IC_{50} value of 0.12 μM. We observe that CaMKIINtide is a competitive inhibitor of *C. elegans* CaMKII with respect to autocamtide (Figure 2.12), as reported by others for the mammalian enzyme (Knighton et al., 1991). Mutational analysis has indicated that CaMKIINtide interacts with docking site B (Vest et al., 2007), but the primary sequence of CaMKIINtide does not indicate an obvious mode of inhibition.

We determined the crystal structure of a 21 residue segment of CaMKIINtide bound to the kinase domain of CaMKII at 3.4 Å resolution, and observe a mode of interaction that is very similar to that observed for the *in trans* interaction of the R1 element (Figure 2.11). Instead of threonine, the inhibitor has an Arg residue at the P0 site, and therefore cannot be phosphorylated. Several hydrophobic and basic residues are present in the N-terminal portion of the inhibitor, and confer specificity for the CaMKII kinase domain by using interactions in docking sites B and C (Figure 2.11). The visualization of a mode of inhibition that is similar to the R1 interaction suggests that CaMKIINtide, in addition to inhibiting the activated subunit, would also block the capture of adjacent regulatory segments. While CaMKIINtide can block peptide substrate phosphorylation, it apparently does not block autophosphoryation (Vest et al., 2007), suggesting that high CaMKIINtide concentrations would be necessary to block inter-subunit capture interaction from occurring within the holoenzyme. At sub-saturating levels of inhibition, it is conceivable that different expression levels of CaMKIINtide could tune the cooperative response of CaMKII.

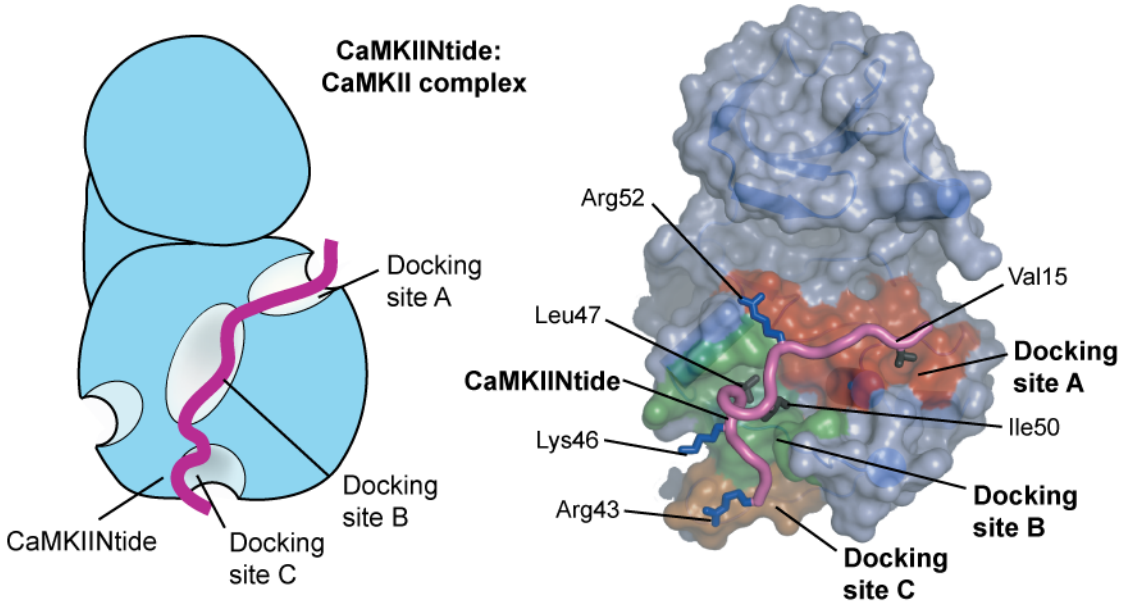


Figure 2.11 Structure of the CaMKII inhibitor CaMKIINtide bound to the kinase domain

At left is shown a schematic diagram of the CaMKII kinase domain and the docking regions occupied by CaMKIINtide. At right, a surface representation of the CaMKII kinase domain with the CaMKIINtide peptide bound is shown, with critical residues highlighted. Basic residues (Arg 43 and Lys 46 (rat numbering for CaMKIINtide(Chang et al., 2001)) occupy docking site C and B, Leu 47 and Ile 50 occupy docking site B, and a pseudosubstrate recognition mode of interaction is observed in docking site A (Arg 52 at the P–3 site, Val 56 at the P+1 site).

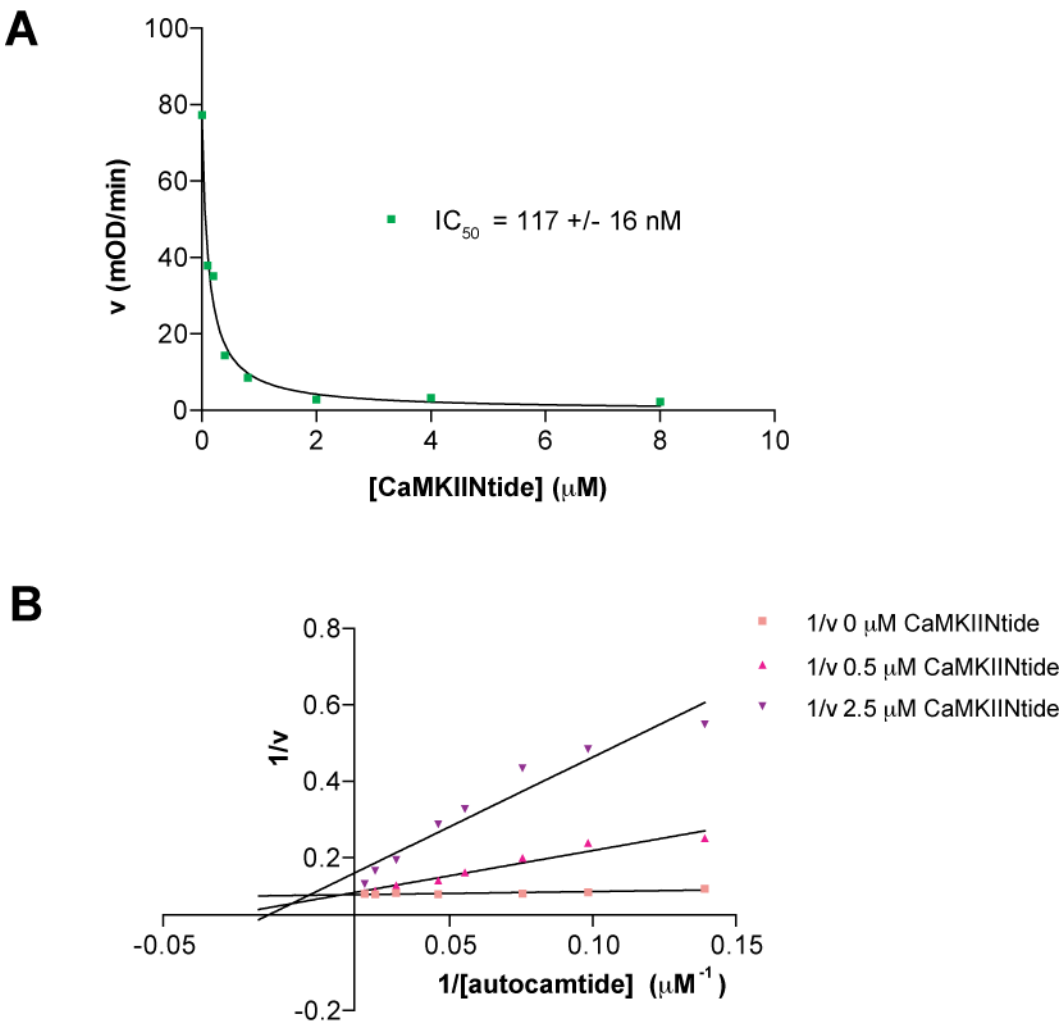


Figure 2.12 CaMKIINtide inhibition of CaMKII is competitive for autocamtide

A. The activity of CaMKII kinase domain (residues 1-277) towards syntide was measured at varying concentrations of CaMKIINtide peptide (27-mer comprising residues 42-68 of CaMKIIN α) (top panel). **B.** The measured activity of CaMKII kinase domain (1-277) while varying the concentration of autocamtide indicates a competitive mode of inhibition.

2.3 Conclusions

The observation that the activation of CaMKII by Ca^{2+}/calmodulin is cooperative means that adjacent kinase domains are activated preferentially over those located more distantly within the holoenzyme. This rules out a simple coincidence detection mechanism such as the one depicted in Figure 2.1 A. Our previous analysis of the structural basis for cooperativity in CaMKII focused on the role of autoinhibited kinase domain dimers in the process (Rosenberg et al., 2005). If the binding of two molecules of Ca^{2+}/calmodulin affects only two kinase subunits, then the value of the Hill coefficient should be between 1.0 and 2.0. Our new data, which show that the Hill coefficient for activation is greater than 2.0 for many CaMKII constructs suggests that there are additional interactions between dimers in the holoenzyme, such that disruption of one dimer facilitates the disruption of additional dimers.

A reduction in the Hill coefficient is observed when autocamtide, the peptide derived from the regulatory segment, is used as a substrate. For the construct with the greatest degree of cooperativity (CaMKII Δ17, see Fig. 2.2 C), the Hill coefficient is reduced from ~4.0 with syntide to ~1.8 with autocamtide. This is consistent with a role for autocamtide in weakening interactions between dimers. Our finding that the R1 element of the regulatory segment makes extensive interactions with another kinase domain in a *trans* configuration suggests that an activated kinase domain can capture the regulatory segment of an adjacent kinase domain, potentiating the binding of Ca^{2+}/calmodulin to it. This is supported by the results of experiments in which a decoy kinase domain provide open binding sites for the regulatory segment, resulting in cooperativity for the activation process. The higher affinity of autocamtide compared to syntide for the substrate binding site is expected to impair the efficiency of this process.

Our results bring to the forefront the question of how an appropriate time delay is introduced in the transphosphorylation of Thr286, a critical parameter in determining the sensitivity of the CaMKII holoenzyme to the frequency of Ca^{2+} spikes (De Koninck and Schulman, 1998). Resolution of this issue awaits further experimentation, but one clue is provided by dramatic structural differences between two states of CaMKII that have been noted in electron microscopic reconstructions from different groups (Kolodziej et al., 2000; Morris and Torok, 2001). In one set of reconstructions the kinase domains appear to be arranged in the central plane of the holoenzyme assembly (Morris and Torok, 2001). This is consistent with SAXS analysis of inactive CaMKII (Rosenberg et al., 2005), which led to a model in which dimeric autoinhibited kinase domains form an outer ring around the central hub of association domains. In another set of electron microscopic reconstructions the kinase domains are located in two rings, one well above the central plane, and one below it (Kolodziej et al., 2000).

We speculated previously that Ca^{2+}/calmodulin binding and the subsequent disruption of kinase domain dimers might result in these domains moving apart from each other, above and below the association domain ring (Rosenberg et al., 2005). This is consistent with the increased distance between kinase domains that results from activation, as inferred from FRET measurements (Thaler et al., 2009), and with the second set of electron micoscopic reconstructions (Kolodziej et al., 2000). In such a mechanism for setting an autophosphorylation time delay, the first pair of kinase domains that are activated are prevented from phosphorylating each other because they are located on opposite sides of the central plane (Figure 2.13).

Transphosphorylation would await the release of the next pair of kinase domains, aided by the substrate capture mechanism that is suggested by our data. A clearer picture as to how the timing of this step is determined will emerge after the kinetic rate constants for the various steps are measured. One exciting possibility for the future is that the frequency-dependent process may be monitored directly in neuronal dendrites, as highlighted by a recent study on the *in situ* activation of CaMKII by laser stimulated Ca^{2+} spikes (Lee et al., 2009).

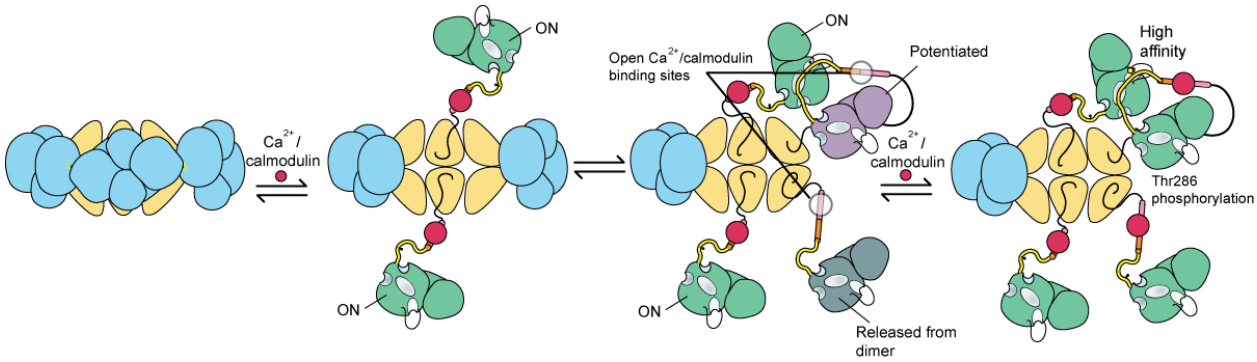


Figure 2.13 A hypothetical mechanism for CaMKII activation

A hypothetical mechanism for CaMKII activation. If the time interval between Ca^{2+} spikes is long compared to the dissociation time of Ca^{2+} calmodulin (low frequency), initial binding are presumed to lead to a separation of kinase domains, however transphosphorylation of Thr286 does not occur before Ca^{2+}/calmodulin dissociates. If the interval between Ca^{2+} spikes is short compared to the Ca^{2+}/calmodulin dissociation time (high frequency regime). Ca^{2+}/calmodulin remains bound to the first pair of kinase domains long enough for a second, slow, step to occur in which the activated kinase domains capture the regulatory segments of adjacent kinase domains. This potentiates the binding of Ca^{2+}/calmodulin to those domains with increased affinity, resulting in the phosphorylation of Thr286 and acquisition of autonomy (Ca^{2+}/calmodulin-independent activity).

2.4 Materials and methods

2.4.1 Protein expression and purification

We developed a bacterial expression system for CaMKII by coexpression with Lambda Phosphatase, using a strategy similar to that for bacterial expression of certain tyrosine kinases (Seeliger et al., 2005). We obtained yields of 2.5 mg of pure active holoenzyme per liter of bacterial culture, compared to 5-10 mg per liter of insect cell culture(Rosenberg et al., 2005). Bacterial expression of CaMKII in *E. coli* was accomplished by coexpression with lambda phosphatase (kind gift of J. Dixon, University of California, San Diego) in Tuner(DE-3)pLysS cells (Novagen). Lambda Phosphatase was subcloned into a pCDFDuet1 vector (Novagen) and C-terminally 6-histidine tagged CaMKII and its mutants forms were cloned into a pET-20b vector (Novagen). Protein expression in bacteria was induced by the addition of 0.4 mM IPTG and 0.5 mM Mn^{2+}. Cells were grown overnight at 20^{o}C and flash frozen until used. The average yield was 2 mg of protein per liter of cells at >95% purity as judged by SDS/PAGE and Comassie stain. Autoinhibited constructs of CaMKII (residues 1-340), and other truncations (residues 1-274 and residues 1-291) of the *C. elegans* unc-43 gene were fused to an N-terminal cleavable 6-histidine tag and cloned as described previously.

Our experiments focused on the *C. elegans* enzyme because crystal structures were determined initially using this enzyme (Rosenberg et al., 2005). Key experiments are repeated using mammalian enzyme, from *Sus scrofa*. Crystal structures for truncated forms of the human α, β, δ and γ isoforms of the human CaMKII autoinhibited kinase domain, determined by the Structural Genomics Consortium, are also available (PDB codes 2VN9, 2VZ6, 2V70 and 3BHH). The sequence identity within the kinase domain and the association domain between the *C. elegans* and human enzymes are ~80% and ~50%, respectively.

Purification of the CaMKII holoenzyme and the kinase domain constructs was accomplished by Ni-NTA affinity chromatography using a HiTrap HisBind column (Pharmacia) followed by anion exchange and S200 size exclusion chromatography. The final buffer from the gel filtration was 25 mM tris(hydroxymethyl)aminomethane hydrochloride (pH 8.3), 250 mM potassium chloride, 10% (v/v) glycerol, and 1mM tris(2-carboxyethyl)phosphine. Wild type, full-length *C. elegans* CaMKII (encoded by the unc-43 gene) was also expressed in Sf9 cells using a baculovirus expression system as described previously(Rosenberg et al., 2005). The enzyme purified from the bacterial source, used in the experiments described here, behaves similarly to that purified from insect cell culture, as assessed by mass spectrometry, analytical gel filtration and enzyme activity assays (data not shown). *Gallus gallus* calmodulin was expressed and purified as previously described (Putkey and Waxham, 1996) and its final concentration was determined by amino acid analysis (Molecular Structure Facility, University of California, Davis). The *S. scrofa* γ isoform of CaMKII was expressed in Sf9 cells using a baculovirus expression system (Rosenberg et al., 2005) and purified as described above.

2.4.2 Crystallographic analysis

Crystals of CaMKII (residues 1-289) were grown using sitting drop vapor diffusion. Crystals were obtained with 0.1 M potassium chloride, 5 mM magnesium sulfate, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.0 and 15% (v/v) 2-methyl-2,4-pentanediol. Crystals of CaMKII (1-272) and CaMKIINtide were grown in 20% (v/v) 2-methyl-2,4-pentanediol. Crystals were cryoprotected in 25% (v/v) glycerol prior to being frozen in liquid nitrogen. X-ray diffraction data were collected at the Advanced Light Source beamline 8.2.2 at 100°K, at the wavelength .9537Å.

Structures were solved by molecular replacement using Phaser(McCoy et al., 2007), using the autoinhibited kinase domain of CaMKII (residues 1-278) as a search model. Refinement was performed with CNS (Brunger et al., 1998) and PHENIX (Adams et al., 2002) and model building with O (Jones et al., 1991) and Coot (Emsley and Cowtan, 2004).

The coordinates for structures solved in this study were deposited in the Protein Data Bank with ID codes 3KK8 (substrate complex crystal form A), 3KK9 (substrate complex crystal form B), 3KL8 (CaMKIINtide complex).

2.4.3 Enzyme activity assays

Kinase activity was monitored using a continuous spectrophotometric assay as described earlier (Barker et al., 1995). In this assay, ADP that is produced as a result of phosphorylation by the enzyme is coupled to the oxidation of NADH to NAD^+, which produces a decrease in absorbance at 340 nm. The assays were carried out in 100 mM tris(hydroxymethyl)aminomethane hydrochloride (pH 7.5), 150 mM potassium chloride, 0.2 mM calcium chloride, 10 mM magnesium chloride, 0.5 mM adenosine 5'triphosphosphate, 1 mM phosphoenolpyruvate, 0.28 mM nicotinamide adenine dinucleotide, 89 units/ml pyruvate kinase, 124 units/ml lactate dehydrogenase, 0.3 - 0.5 mM peptide substrates autocamtide AC-3 (KKALHRQETVDAL) or syntide (PLARTLSVAGLPGKK) and various concentrations of calmodulin, at 30°C in a 150 µl reaction volume. Peptides were prepared by David King (Howard Hughes Medical Institute). Reactions were initiated by the addition of 10 - 20 nM CaMKII to the mix and the decrease in absorbance was monitored at 340 nm at 30°C in a microtiter plate spectrophotometer (SpectraMax). The enzyme concentration is expressed in terms of the concentration of kinase units and not holoenzymes. In addition, CaMKII activity was also measured using a phosphocellulose filter binding assay and ATP-^{32}P as previously described (Bradshaw et al., 2003).

Cooperativity curves were plotted and analyzed using the program Prism (version 5, GraphPad Software). The data was fit to the Hill equation:

$$Y = Y_{\min} + \frac{(Y_{\max} - Y_{\min})}{1 + \left(\frac{10^{(\log_{10} EC_{50})}}{10^{(\log_{10} [L])}} \right)^n}$$

Where Y is the maximal velocity, EC_{50} is the concentration at half maximal velocity, [L] is the ligand concentration of calmodulin, and *n* is the apparent Hill coefficient.

Chapter 3:

Exchange of CaMKII subunits

3.1. Overview

Cellular signal transduction utilizes protein components that have limited life-spans; most signaling proteins are degraded within hours (Varshavsky, 1997). Nevertheless, biological processes occur over much longer periods of time. Most notably, memories formed in the brain remain for tens of years, and the causative cell signaling events that result in long-term changes in synaptic strength are observed to last from days to weeks. It remains unknown how transient protein species are capable of producing long-lasting signals, especially for important processes such as learning and memory.

Francis Crick considered several solutions to the problem of molecular turnover and memory (Crick, 1984). One possibility is the coding of memory in the epigenetic state of DNA. This would produce a stable 'mark' of previous events in some form of nucleic acid. Since this mechanism would require a specialized piece of nucleic acid at each synapse, Crick considered this mechanism as unlikely. Another possibility is a molecule that is relatively immune to protein turnover, which he proposed could be investigated through some type of pulse-chase experiment. This possibility was also not considered probable, however. Crick proposed a most intriguing solution to the problem of molecular turnover and memory: the presence of "...molecules in the synapse [which] interact in such a way that they can be replaced with new material, one at a time, without altering the overall state of the structure" (Crick, 1984). In this model, some type of reversible modification within an oligomer enables a molecule to exist in two states: active and inactive. Crick set two important conditions for the system: first, that active monomers are capable of converting inactive monomers within oligomer into the active state; second, that newly synthesized unmodified monomers are able to be substituted into existing oligomers. This property of molecular turnover, or exchange of subunits between oligomers, would enable a signal to outlast constant metabolic turnover (Figure 3.1).

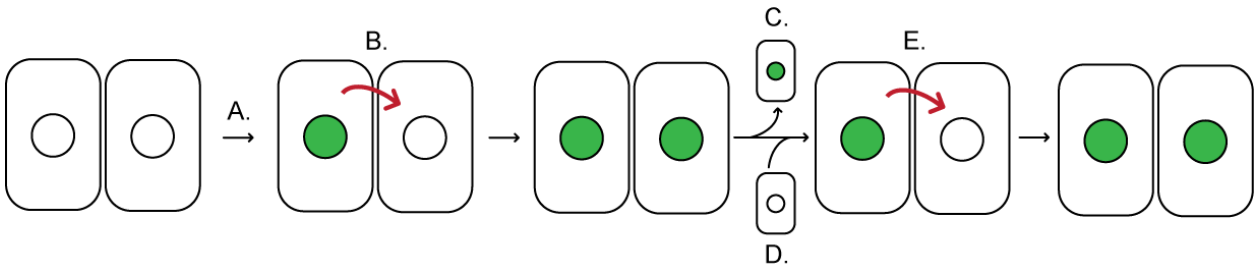
John Lisman observed that Crick's conditions for resistance to molecular turnover could also be satisfied by a bi-stable kinase switch (Lisman, 1985), and that inter-molecular phosphorylation could produce a system which produced a signal insensitive to protein degradation. Lisman noted that CaMKII might have many of the properties necessary for signal maintenance (Lisman, 1994). First and foremost, the enzyme is an oligomer of identical monomers. In addition, the enzyme is activated by calcium/calmodulin binding on adjacent subunits, and transphosphorylation may occur as an inter-molecular reaction. Together, these observations have led to the proposal that learning and memory might be enabled, in part, by signal maintenance mediated by CaMKII subunit exchange. We set out to investigate whether CaMKII can undergo subunit exchange, and if so, what effect this might have on the activity of the enzyme.

A model of the CaMKII holoenzyme, based on structures of CaMKII association domain oligomers and structures of kinase domain dimers, provides a physical basis for evaluating whether CaMKII is capable of maintaining a signal resistant to protein turnover (Hoelz et al., 2003; Rosenberg et al., 2006). The major interactions necessary for assembly of the holoenzyme occur between the association domains. Each association domain is composed of a single α-helix supported by an anti-parallel β-sheet. The association domains form a central hub-like assembly of two stacked hexameric rings from which the kinase domains protrude (Figure 3.2). Two sets

of inter-association domain interactions can be observed. First, within each hexameric ring, the association domains are arranged in a head-to-tail fashion, stabilized by a mixture of hydrophobic and salt-bridge interactions. In all discussion that follows, I shall refer to this set of interactions as the intra-ring interface. Second, between the two hexameric rings, pairs of stacked β-sheets link adjacent association domains to form an extensive interaction surface. This set of interactions will be referred to as the β-sheet interface. The β-sheet interface interaction surface is more extensive (1100 $Å^2$) than the intra-ring interface, and much of this interaction surface is polar and contains several histidine residues.

As a first step in understanding the potential for exchange of CaMKII subunits in the maintenance of cellular signals, I used Förster Resonance Energy Transfer (FRET) to look for exchange of CaMKII subunits between holoenzymes. I found that indeed, CaMKII subunits are competent to exchange, and that this process occurs both in the context of isolated association domain oligomers, and in the context of the phosphorylated holoenzyme. I hypothesized that the mechanism of subunit substitution likely involves the exchange of subunit dimers, and that the intra-ring interfaces may be the sites of subunit insertion and removal. To test the effect of altered subunit affinity on subunit exchange, I constructed a fusion protein consisting of CaMKII and Hcp1, a protein known to exist at a stable hexamer, and measured subunit exchange using a similar assay. I found that oligomers of the CaMKII-Hcp1 fusion protein exchanged subunits between complexes at a dramatically reduced rate. These data suggest that oligomeric assemblies are not fixed, but instead undergo subunit exchange, supporting the hypothesized role for subunit exchange in prolonging the lifetime of a phosphorylation signal in neurons.

Figure 3.1 A Mechanism for a "Molecular Memory" of a signal resistant to protein turnover.



A schematic depicting a possible mechanism for molecular memory proposed by Francis Crick. Illustrated is a dimeric system for simplicity. In such a system, subunits may exist in an inactive (white) or active (green) state. **A.** An initial stimulus activates a subunit (indicated by green dot). An important property of this system is that active subunits can convert inactive subunits within an oligomer, indicated as step **B.** Over time, subunits are degraded (**C**), however, if newly synthesized subunits are able to exchange into complexes containing previously activated subunits (**D**), subsequent trans-subunit activation enables the signal to remain maintained (**E**).

3.2 Results and discussion

3.2.1 CaMKII association domains undergo subunit exchange

Crystal structures of the association domain of CaMKII are available from human (γ isoform), *C. elegans* and mouse (α isoform) (homologues (PDB codes 2UX0, 2F86, 1HKX respectively) (Hoelz et al., 2003; Rosenberg et al., 2006). Despite being from different isoforms, the residues at the β-sheet interface and the intra-ring interfaces between association domain interfaces are identical, and the interfaces are superimposable. An intriguing feature of the first association domain structure solved (that of mouse α) was that a tetradecameric assembly was observed (Figure 3.2 A). Subsequent electron microscopy analysis of full-length CaMKII and isolated association domain oligomers suggested that the kinase domains are involved in maintenance of the dodecameric form of the assembly (Rosenberg et al., 2006). Other association domain structures have illustrated that the association domain can also be crystallized in the dodecameric form (PDB code 2UX0, Figure 3.2). The ability to observe 12-mer and 14-mer forms suggests that the assembly is dynamic.

Crystal structures of two CaMKII association domain orthologues from the bacteria *Exiguobacterium sibiricum 255-15* and *Thiobacillus denitrificans* have been solved (PDB codes: 3KSP and 3GWR, respectively). Both of these bacterial association domains display a similar fold comprising a single α-helix cradeled by a β-sheet. Intriguingly, while these association domains display a dimeric assembly in the crystal lattice, similar to that seen in the metazoan forms, the residues at the intra-ring interface important for mediating the hexameric association assembly are not conserved, and these bacterial association domain orthologues do not display a higher order assembly beyond a dimer in the crystal lattice (Figure 3.3). Thus, I wondered if the ability of association domains from CaMKII and related proteins to adopt different oligomeric states was indicative of an inherent ability of the domain to undergo subunit swapping between complexes.

I postulated that if subunit exchange between holoenzymes were to occur, the hexameric interface would be a likely region where subunits could be substituted. The tetradecameric association domain crystal structures could represent an intermediate in the exchange process, which would be transient or unstable in the holoenzyme state due to the unfavorable steric clashes in packing the of kinase domains, thus favoring a dodecameric form. To evaluate the ability of subunits to exchange between association domain complexes, I labeled two separate pools of association domains at an endogenous surface-exposed Cys residue (Cys 366). One pool was labeled with Alexa Fluor 488, and the other pool was labeled with Alexa Fluor 594. A labeling efficiency of ~80% was achieved. I monitored first the emission of the acceptor dye (at 617 nm) over time, while exciting at the donor excitation wavelength, 488 nm. No major change in fluorescence intensity was observed. Unlabeled association domain was then added, and again, no change in fluorescence intensity at 617 nm was observed. When a population of association domain labeled with a donor fluorophore (Alexa Fluor 488) was added, an increase in fluorescence intensity was observed, attributable to FRET between mixed association domains containing both donor and acceptor fluorophores. This result indicated that association domains labeled with donor fluorophores were exchanging into oligomers of association domains labeled with acceptor fluorophores.

Subunit exchange is the second critical condition required for the Crick mechanism for molecular memory. A process attributable to subunit exchange was observed by FRET to occur on a timescale of minutes. Since the rate of the observed exchange process is faster than the average lifetime of protein species (hours), these experiments observe a process fast enough to overcome protein degradation. These experiments observe exchange between complexes of the isolated assocation domains. Due to the absence of the kinase domains, one can infer that this process occurs independent of any inter-kinase domain interactions, and is an intrinsic property of the association domain assembly. An increase in FRET signal due to the close proximity of labeled subunits could reflect a process of aggregation, or interactions between multiple association domain assemblies. I monitored the elution of isolated association domain species by gel-filtration coupled to multi-angle light scattering, and found that these samples are monodisperse and elute at a single peak reflective of a tetradecameric assembly (data not shown), suggesting that aggregation or inter-assembly interaction is not responsible for the observed FRET changes. These experiments open new questions regarding mechanisms for subunit exchange between CaMKII association domains. It remains an open question whether monomeric subunits are the only form competent for subunit exchange, or if dimers and higher order forms of the association domain are stable to swap between association domain assemblies.

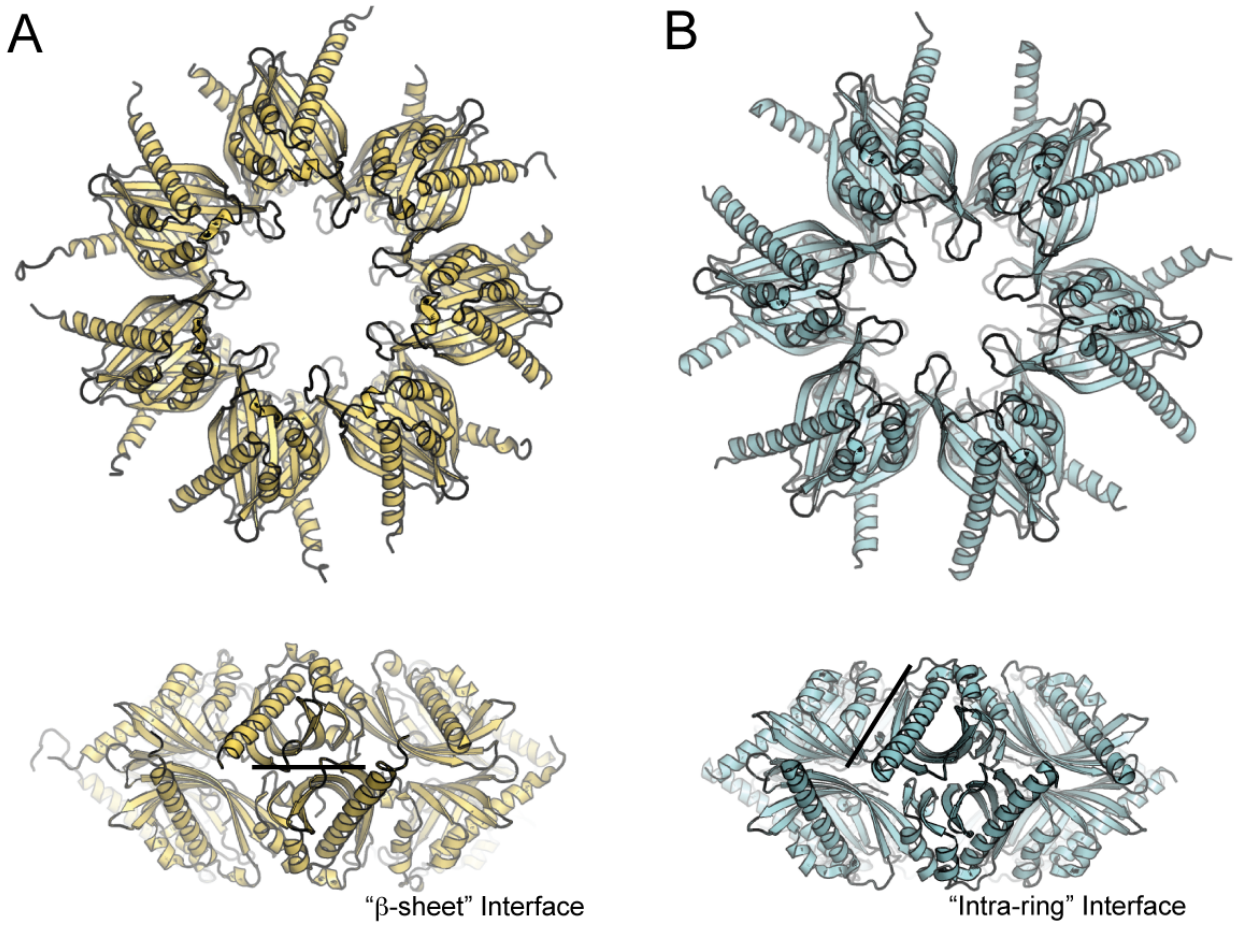
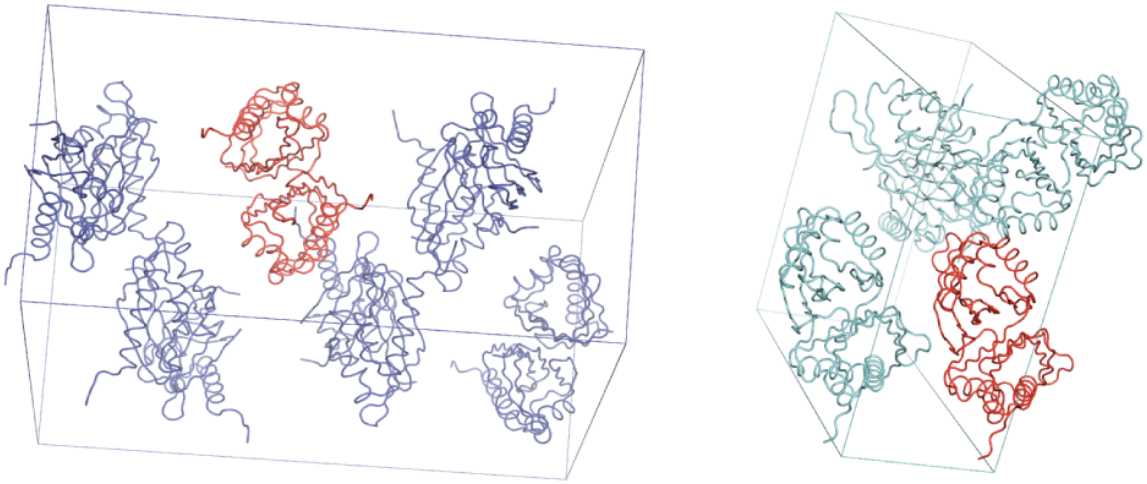


Figure 3.2 The CaMKII association domain displays variability in its oligomeric state.

A. The tetradecameric association domain assembly from the mouse α CaMKII isoform (PDB code 1HKX) (Hoelz et al., 2003) **B.** The dodecameric association domain assembly from the human gamma CaMKII isoform (PDB code 2UX0). Shown is the approximate location of the two interfaces that mediate the association domain assembly (black line).

A



B

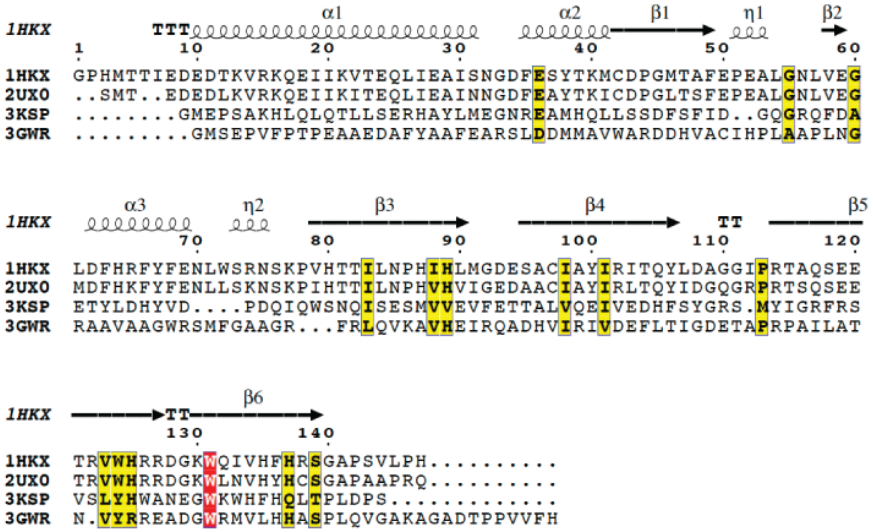


Figure 3.3 Orthologues of the CaMKII association domain do not display a dodecameric or tetradecameric oligomeric arrangement in the crystal.

A. Crystal packing of other CaMKII orthologue association domains. Shown is the crystal packing of two orthologues from *Exiguobacterium sibiricum 255-15* (left) and *Thiobacillus denitrificans* (right) (PDB codes 3KSP and 3GWR, respectively), which were crystallized in space groups $P6_522$ and $P2_12_12_1$, respectively. Both show dimeric arrangements within the crystal, however no higher order assembly beyond a dimeric species is observed.

B. Sequence alignment of these orthologues with metazoan forms.

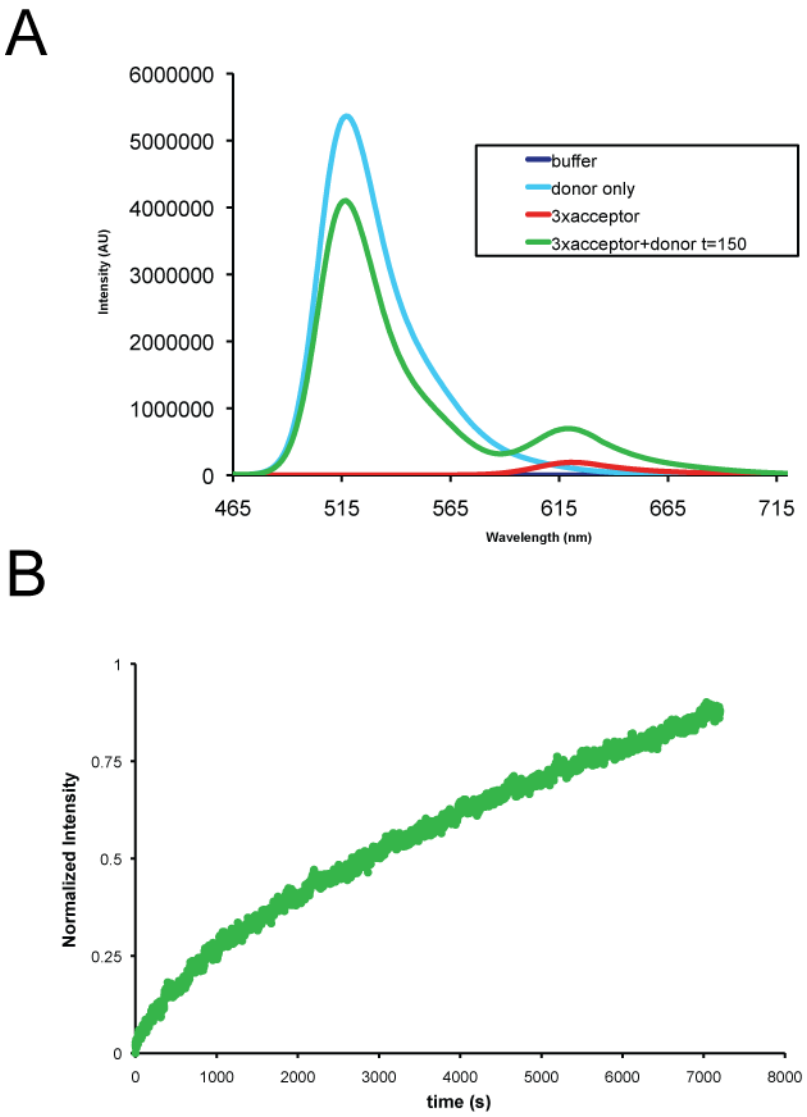


Figure 3.4 CaMKII association domains undergo subunit exchange.

A. Florescence spectra of CaMKII association domain samples. Shown are emission spectra of samples excited at 488 nm. In dark blue is the spectra of buffer alone, in cyan is the spectra of the donor Alexa Fluor 488-labeled CaMKII construct alone, in red is the spectra for the Alexa Fluor 595-labeled CaMKII construct alone, and in green is the spectra of Alexa Fluor 488-labeled CaMKII with 3-times molar excess acceptor-labeled association domain after 150 seconds.

B. Show is the time evolution of FRET increase, as shown as the increase in fluorescence intensity at the acceptor emission wavelength, 618 nm.

3.2.2 Phosphorylated CaMKII holoenzymes undergo exchange

Since I observed FRET changes attributable to exchange of subunits between association domain complexes, I wished to know if exchange occurred between CaMKII holoenzymes as well. To evaluate this, I labeled two separate pools of full-length CaMKII holoenzyme on four endogenous surface-exposed Cys residues using maleimide Alexa Fluor 488 and Alexa Fluor 595. High-efficiency labeling was achieved using either dye, with approximately ~3 dye molecules per subunit of CaMKII. An increase in FRET signal was observed for full-length holoenzymes, and this process had a shorter half-life (~20 minutes) than that for the association domain (~45 minutes) (Figure 3.5 A). This observation indicates the presence of the autoinhibited kinase domains in the full-length assembly alters the association domain exchange properties. One possible interpretation is that a complex containing only the association domain is a more stable complex, and that the full-length holoenzyme complex containing kinase domains is more prone to the exchange of subunits.

I postulated that exchange occurred through swapping of association domains between holoenzymes through the intra-ring interface. To evaluate this possibility, I constructed a fusion of CaMKII and a known stable hexameric protein, Hcp1. Hcp1 is a bacterial beta-barrel protein that has been observed to form a stable hexamer by electron microscopy and by X-ray crystallography (PDB code 1Y12) (Mougous et al., 2006). The fusion construct consisted of Hcp1 attached to the C-terminus of the association domain of full-length CaMKII. This construct was labeled in a similar manner, and the exchange of these subunits was evaluated by the FRET assay. The Hcp1 holoenzyme shows much less FRET change compared to the association domain and full-length holoenzyme constructs (Figure 3.5). The reduction in FRET signal in the Hcp1-fusion indicates that the intra-ring interface may be stabilized by Hcp1, thus slowing exchange. Inter-holoenzyme interactions have been proposed to enable kinase subunit-mediated interactions between holoenzyme complexes, and could theoretically account for our observed FRET signal change (Hudmon et al., 2005). However, this is unlikely because in the experiments presented here, the association domain construct (lacking the kinase domains) undergoes the FRET increase which reflect exchange, and the Hcp1 fusion show reduction in the rate of FRET change, indicating that the FRET signal change can be changed by disrupting the properties of the association domain alone.

An important component of the exchange model is the ability of activated holoenzyme to swap subunits. I tested this possibility by phosphorylating the holoenzyme through incubation of calcium-saturated calmodulin with labeled subunits. I then tested the ability of autophosphorylated holoenzyme to exchange. The phosphorylated CaMKII holoenzyme shows an increase in FRET signal, reflecting subunit exchange, albeit at a rate slower than the unphosphorylated holoenzyme. This rate is similar to that for assocation domains alone. Small angle X-ray scattering studies of CaMKII in the calcium/calmodulin-bound state show that the kinase domains form an extended arrangement, released from compact autoinhibited interactions (Rosenberg et al., 2005). The observation that the rate of exchange of phosphorylated subunts is similar to the rate of exchange of the association domain alone is consistent with the phosphorylated holoenzyme producing a loose assembly of kinase domains. This observation indicates that once kinase domains are released from their autoinhibited state, these subunits exchange between complexes in a similar fashion to complexes of association domains alone.

This suggests that the kinase domains do influence the exchange properties of the association domain, when in the autoinhibited state. One major feature of the autoinhibited kinase domain is a coiled-coil strut formed by the regulatory segments. Since this structure is dissolved upon calmodulin activation, these exchange data suggest that this structural region could play a role in influencing the changes necessary for exchange between association domain assemblies.

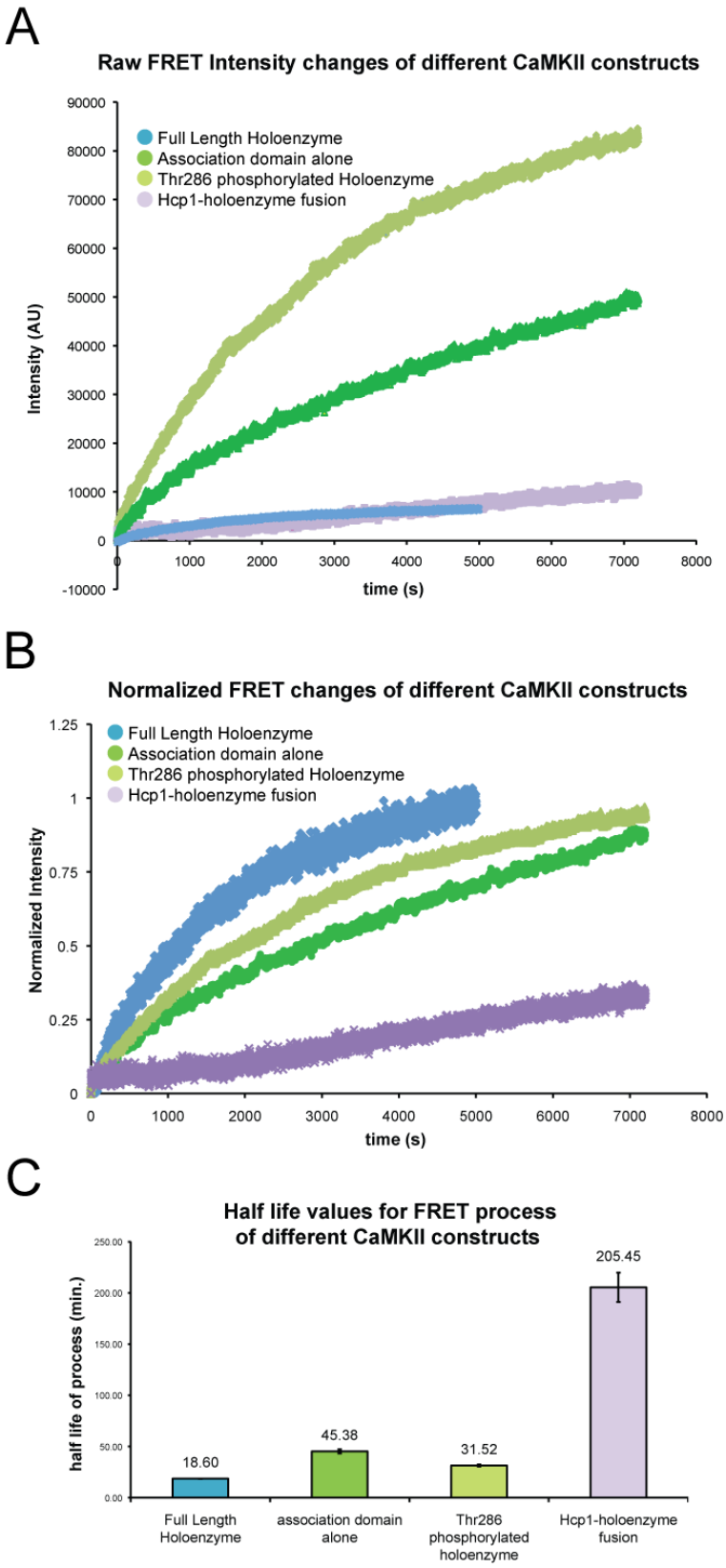


Figure 3.5 Inactive and Phosphorylated CaMKII holoenzymes undergo exchange.

3.3 Conclusions

CaMKII is unique amongst protein kinases due to its oligomeric architecture. Cooperative activation of the enzyme results in autophosphorylation at Thr 286. Following activation of the holoenzyme, the phosphorylation signal could be attenuated by protein degradation. We have investigated the potential for the CaMKII holoenzyme architecture to enable subunit exchange between dodecameric complexes. Such an ability is required if CaMKII functions in maintaining a stable 'biochemical memory' of an activation event.

The data presented here demonstrate that subunits of CaMKII do exchange, and do so on at a time scale of tens of minutes. This may be sufficient to maintain an autophosphorylation signal, as this rate is faster than measured rates for protein degradation. These results demonstrate the biochemical potential for subunit exchange, and satisfy one important condition in the proposed mechanisms of molecular memory. Differences rates of exchange were observed depending on the activation state of the kinase domains. It is not known whether such processes are important for CaMKII's role in neurons, and the implications for different exchange rates for activated compared with inactive holoenzymes remain unknown. Pharmacological and genetic manipulation have indicated that CaMKII is important in the initiation of Long Term Potentaion (Lisman et al., 2002). However, the role for CaMKII in the later stages of LTP remains undemonstrated.

Activation of calcium signaling proteins in neurons is highly specific for different anatomical regions of neurons. This 'local' character of neuronal calcium signaling is thought to be of vital importance for the ability to form a given neuron to form multiple synapses, and for neuronal circuits to be constructed (Sabatini et al., 2002). A critical feature of this local signaling is the sequestration of signaling components in specific neuronal compartments. CaMKII interacts with numerous scaffolding proteins in each neuronal dendrite (Sheng and Hoogenraad, 2007). It is possible that the exchange of subunits may be important for signal maintenance within a compartment. At the post-synaptic density, where concentrations of CaMKII are high, subunit exchange could enable for the signal propagation and maintenance. Future experiments may investigate how many of CaMKII's localization and scaffolding proteins alter its exchange properties.

Several mechanisms of self-perpetuation have been suggested in maintaining the results of neuronal signaling (Si et al., 2010). The *in vitro* experiments presented here suggest that the CaMKII assembly has the biochemical properties necessary for such a process. These observations indicate that dimeric subunits of the enzyme are capable of exchanging between CaMKII holoenzymes. An intriguing observation from these experiments is that the activation state of the kinase domains alters the exchange dynamics. This model provides a structural mechanism for prolonging molecular memory of signaling events. Future experiments may test the effects of biochemically characterized exchange mutants on the maintenance of calcium signals in neurons, such as in events important for maintaining LTP.

3.4 Materials and methods

3.4.1 FRET based-exchange assay

C. elegans CaMKII constructs were prepared as described previously (Chapter 2, Methods). Proteins were desalted in buffer containing 25 mM Tris-HCl, 250 mM KCl, 10% glycerol using a PD-10 fast desalting column (GE Lifesciences). Samples were incubated with 10-fold molar excess of maleimide-Alexa Fluor dyes (Invitrogen), and incubated at room temperature for 1 hr, after which samples were desalted into the identical buffer containing 1 mM TCEP.

Reactions were measured mixing 100 μl of each protein sample at1 μM using a stopped-flow apparatus (RX2000l Applied Photophysics) linked to a Fluoromax-3 fluorimeter (HORIBA Jobin Yvon). Data were fit to a single exponential function using the program Prism 4 (Graphpad Prism, Inc.).

3.4.2 Cloning and Protein Purification

A CaMKII-Hcp1 fusion construct was made by inserting the Hcp1 gene in-frame with the C-terminus of *C. elegans* CamKII, with an intervening 10-residue linker between the two proteins. The Hcp1 gene was a kind gift of J. Mougous (Univ. of Washington).

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