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Engineering a Fluorescent Calmodulin for Use in Complex Models of Cardiac Physiology

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Abstract

Calmodulin (CaM) is a ubiquitously expressed calcium-binding protein that plays a critical role in cardiac physiology. CaM senses intracellular Ca^{2+} changes by binding the Ca^{2+} ion with one of its four calcium-binding loops, causing a conformational change in the CaM protein structure, and thereby affecting CaM's affinity for its multiple intracellular protein targets. Since Ca^{2+} transients rise and fall in the heart on a beat-to-beat basis, an understanding of the kinetics of CaM- Ca^{2+} binding and dissociation events are essential for studying how cardiac systems are regulated. Isolated CaM kinetic experiments have described CaM behavior in controlled solutions, but aren't directly applicable to the protein's complex physiological environment. We employed site-directed mutagenesis to generate three CaM mutants with Threonine to Cysteine substitutions at residues 5, 34, and 44 (T5C, T34C, T44C). A fluorescent probe (IAANS) was covalently linked to the introduced Cysteine residue. These fluorescent Calmodulin proteins have a unique fluorescent behavior that differentiates them from any intrinsic (Tryptophan, Phenylalanine, Tyrosine) fluorescent signals. We demonstrate that two of these proteins (T5C, T34C) have fluorescent behavior that closely mimics that of WT CaM in the presence of representative peptide fragments of Myosin Light Chain Kinase (RS-20), Neural Nitric Oxide Synthase (nNOS), Calcineurin (CaN), and CaM-dependent Kinase II (CKII). Thus, T5C or T34C CaM labeled with IAANS has unique fluorescent properties without drastically affecting the behavior of the CaM protein. These fluorescent proteins give researchers the freedom to study CaM kinetic characteristics in the presence of enzymes regulated by CaM, allowing for more research with direct relevance to physiological pathways.

Engineering a Fluorescent Calmodulin for Use in Complex Models of Cardiac Physiology

Chapter I

Before every NASA rocket launch, before every clinical drug trial, before every building demolition, you can expect one common process. No matter how much is known about projectile motion, pharmacology, dynamite, or otherwise, there is enough room for error that scientists are drawn to model systems to make sure that ideas will work full-scale. So, the NASA engineers turn to computer models to judge the feasibility of new rockets. Doctors have animal models to predict the efficacy and safety of a drug in humans. And, with similar safety concerns in mind, a demolition crew can blow up a small model of a building in an empty field before they implode the real thing near a bustling metropolis. Models give scientists an opportunity to reduce the scale, complexity, or cost of the real thing, so that they can make assessments and adjustments before moving on to more complex tasks.

In the realm of biology, model systems become extremely useful. For example, the behavior of a cell in culture can be used to describe the behavior of a tissue, an organ, a system, and so forth. The behavior of a protein *in vitro* might indicate its function *in vivo*. Biology is particularly well-suited to be modeled, because we can't feasibly understand how organisms work just by observing them macroscopically. The essence of the scientific method revolves around breaking down problems to their simplest form, eliminating confounding variables, and testing specific hypotheses. However, biological systems are not always amenable to experimentation. If a scientist wanted to examine, for example, the mechanisms underlying contractility of the heart, he is confronted with a bevy of ethical concerns. Rightfully so, living things are not to be wastefully expended at the altar of science, unless absolutely necessary to test a specific, important hypothesis.

Like the NASA engineer or the building demolition crew, the biologist is drawn to model systems when designing experiments concerning living systems. Model systems allow the biologist to reduce the complexity of physiological systems, so that he can eliminate confounding variables and better understand his data. A scientist can't expect to master the complexity of the heart by studying the organ intact in animals. Some reductionism must be applied. To understand the myocardium, he might extract and study a single cardiac myocyte. To understand the myocyte, he might analyze the structure and function of a single myosin molecule. It is apparent that a myriad of model systems, all with varying complexity, could be designed to understand the function of the heart.

One player in cardiac function, and the main character of this thesis, is calmodulin (CaM). This small protein is vital for animal and plant life. CaM translates intracellular calcium concentrations into cellular responses. Like any other facet of biology—or science in general—researchers are very interested in designing methods and systems for studying the activity of this protein. Some researchers are trained as physiologists, and hope to characterize the protein as it relates to the living systems it regulates. Other researchers are trained as X-ray crystallographers, and hope to characterize the calmodulin crystal structure. Other researchers still are protein biochemists, and want to understand how CaM's amino acid sequence governs its function. Even though all these researchers are studying the same protein, there are still unlimited approaches to just this one protein.

Problem Statement

In the highly diverse field of calmodulin research, there is a distinct lack of unification between simple and complex model systems. The strategies employed to study isolated CaM in solution are not amenable to study CaM bound to a target, and vice versa. This disjoint prevents

fluidity in translating results from isolated CaM experiments into more complex systems of cardiac physiology. For example, a protein biochemist may discover a way of increasing an isolated CaM molecule's sensitivity to calcium. This is a very exciting proposition for the physiologist that works with CaM, because he can potentially use this sensitized protein to affect the behavior of cardiac muscle. Both researchers need a way of tracking CaM's behavior in their own system, a problem most commonly overcome by fluorescence. The protein biochemist would have made his sensitization discovery using *intrinsic fluorescence*, which is the natural fluorescence of amino acids, such as tyrosine or tryptophan. The physiologist cannot possibly follow the intrinsic fluorescence of CaM in more complex systems—there will be tyrosine and tryptophan residues in virtually every other protein in his model. So the physiologist is more interested in *extrinsic fluorescence*, which are prosthetic fluorescent groups covalently bound to the CaM molecule.

The problem is that the protein biochemist's breakthrough finding might not work in the presence of the physiologist's prosthetic fluorophore. Conversely, discoveries the physiologist might make in a system with extrinsic fluorescence might not be necessarily true for a system using just the isolated CaM protein. In essence, while fluorescence is a common tool for studying CaM function, there is a gap between single-molecule and complex model systems. A fluorescence strategy that is functional for both the isolated protein and also in complex systems involving multiple proteins would facilitate better translation of *in vitro* observations to *in vivo* tests of CaM behavior.

Review of Literature

Properties of CaM. It is logical, before discussing the current status of CaM research, to acquaint the reader with CaM and its role in the human body. Calmodulin is essential for animal

life, and genomic data suggests that evolutionary pressure has ensured that the calmodulin gene is redundant, to avoid lethal recombination events involving the CaM gene (Fischer, et al., 1988). This redundancy might also be attributable for a need for more transcriptional real-estate needed to express high levels of CaM.

CaM is a member of the EF-hand family of proteins, which is a structural motif that consists of a helix-loop-helix pattern. CaM has four such loops, each of which binds a calcium ion, for a total of four calcium binding loops. The protein takes on a dumbbell shape in the crystal structure, as shown in Figure 1. Generally speaking, CaM translates intracellular changes in the calcium concentration into a cellular response by binding calcium and activating targets (Chin & Means, 2000).

When CaM binds calcium ions, it undergoes a conformational change, which is the method by which it is able to regulate other proteins. The surface of the CaM molecule is generally hydrophilic in the absence of calcium. Upon binding calcium, CaM exposes a hydrophobic pocket, and is able to associate with target proteins. These targets are extremely diverse, and can include phosphatases, kinases, and many other types of enzymes. Thus, CaM doesn't have an extremely specific binding region; it is instead the calcium sensor for hire, so to speak. By having flexible binding domain, CaM is able to turn on hundreds of different enzymes (Vogel, 1994).

If the amino acid sequence for each enzyme regulated by CaM were compared to each other, one might expect that the CaM-binding region of each enzyme would be very similar. This, surprisingly, is not the case. CaM does not need to bind to a specific amino acid sequence, which explains why CaM can be such a ubiquitous regulator of enzymatic activity. Enzymes that bind with CaM have a binding site that is basic and hydrophobic. This, in combination with

CaM's hydrophobic, methionine rich flexible interior, facilitates a favorable interaction (Vogel, 1994). Taking all this into account, it seems reasonable that slight alterations can be made to CaM, without necessarily disrupting its ability to be a malleable protein that nonspecifically associates with a legion of different targets. CaM's plasticity will be a characteristic of great importance later in this essay.

Current methods for studying CaM using fluorescence. As mentioned previously, current methodology for studying CaM is a segmented field of study. One sphere of CaM research involves intrinsic fluorescence. This technique is only useful when certain criteria are met. For one, if you're following the fluorescence of a specific amino acid, such as tyrosine or phenylalanine, the more of that amino acid there is in the protein, the more convoluted your signal will be. Figure 2 gives the amino acid sequence of human calmodulin, with residues pertinent to this thesis specially indicated. Notice that calmodulin has eight phenylalanine (F) residues, one tyrosine (Y) residue, and zero tryptophan (W) residues. This relatively low amount of fluorescently active amino acids makes CaM an attractive protein to monitor via intrinsic fluorescence. But, this technique can only be taken so far, because once CaM's binding partners are included in experiments, multiple other fluorescently active residues from the new proteins will override signals from CaM. Thus, intrinsic fluorescence cannot easily be used in any system where CaM is not the lone protein.

Without any genetic modifications, naturally occurring, fluorescently active residues can be monitored, and the conformational changes that CaM undergoes when binding calcium affect the fluorescence of those residues. Thus, researchers can observe calcium binding by observing fluorescence changes. More specifically, the C-terminal half of the protein, which is the only region that has a tyrosine residue, can easily be monitored by selecting for tyrosine fluorescence

properties. Using intrinsic fluorescence, it has been shown that the two domains of CaM—the C-terminal half, and the N-terminal half—have separate calcium binding properties. More specifically, the C-terminal domain has a higher affinity for calcium than the N-terminal domain (VanScyoc, et al., 2002). Of particular interest in the VanScyoc study is the regional specificity of intrinsic fluorescence. Meaning, since tyrosine is only expressed in the C-terminal end of the protein, it is only sensitive to changes that occur in that half of the protein. The idea that fluorescent probes best report activity in their immediate local environment is a crucial aspect of this thesis.

Intrinsic fluorescence does not necessarily imply that the WT protein must be studied. It instead denotes that the fluorescence source is an amino acid within the protein of interest, not a covalently attached fluorophore. That being said, some genetic modifications have been used to better optimize intrinsic fluorescence within CaM. Instead of taking the fluorescent amino acids in a protein for granted, genetic engineering techniques can be used to replace certain amino acids. This is particularly of interest for CaM, because there are no naturally occurring tryptophan (W) residues in the CaM sequence. Thus, by engineering a tryptophan in the place of a phenylalanine, a researcher can selectively monitor specific regions of CaM by choosing where to insert the tryptophan residue. This method has been used to elucidate the magnesium and calcium binding mechanics of CaM (Tikunova, Black, Johnson, & Davis, 2001).

When intrinsic fluorescence is used as a detector, however, other proteins can't be included with CaM in the design of more sophisticated experiments. As detailed earlier, the intrinsic fluorescence properties of each participant protein will conflict, yielding convoluted and uninterpretable data. Although it might not have been much concern, the VanScyoc group was beginning to run into this problem in a 2006 study. Their study was interested in the effects of

point mutations of the CaM sequence, and how those mutations affected CaM calcium binding behavior. A few point mutations identified in *Paramecium* that seemed to impart special characteristics to CaM regulatory activity were tested *in vitro*. Of the dozen or so mutations tested, a few showed alterations in the calcium binding properties of the isolated protein. However, the other mutations did not show differences in calcium binding when compared to control, wild-type (WT) protein. The end of the study surmises that “some of these mutations alter calcium-binding directly, others probably alter CaM-channel association or calcium-triggered conformational change in the context of a ternary complex with the affected ion channel” (VanScyoc, Newman, Sorensen, & Shea, 2006). In this example, the group is so very close to establishing a mechanism for how point mutations in CaM cause differential regulation of ion channels in *Paramecium*. However, they can only test the *in vitro* isolated CaM behavior, and not their hypothesis about ternary complex formation. They’ve hit the ceiling of intrinsic fluorescence—they can’t follow what CaM is doing in the presence of other proteins, they can only test the effects of point mutations on the CaM protein by itself.

On the other side of the rift, there is an entirely different field of CaM research that relies on extrinsic fluorescence. Extrinsic fluorescence, unlike intrinsic fluorescence, does not rely on the natural absorption or emission properties of the WT protein. Instead, fluorescently active molecules called fluorophores are covalently linked to a protein of interest. In order for the fluorophore to be useful, the fluorescence of the fluorophore needs to change as the conformational structure of the protein changes. Thus, a fluorescent signal change is equal to a protein shape change. In calmodulin’s case, a fluorescent signal change is equal to a binding event. That binding event can be CaM binding with calcium, or CaM binding with a target enzyme or peptide.

One possible method for analyzing CaM conformational changes via extrinsic fluorescence utilizes Fluorescence Resonant Energy Transfer (FRET). FRET techniques usually rely on two fluorescent probes being attached to the same protein. The fluorescence of the acceptor molecule is directly related to its distance away from the donor molecule. As the protein changes its conformational shape, the FRET probes move closer or further away, which alters the acceptor's fluorescence. In the field of CaM research, FRET has been used to analyze the characteristics of CaM binding to target proteins. CaM labeled with a FRET probe system was shown to bind to a nitric oxide synthase (NOS) peptide segment with lower affinity than CaM binds to the intact holo-enzyme form of NOS. These results suggest that distal domains of CaM target proteins—not just the CaM-binding regions—are involved in the recruitment and association with CaM (Spratt, Taiakina, Palmer, & Guillemette, 2008). A problem with this FRET-based approach, however, stems from the size of the FRET fluorophores. It is unclear whether or not FRET molecules can accurately measure the activity of a small protein like CaM without altering CaM's characteristics.

Alternatively, FRET probes can also be placed on CaM's target, rather than CaM itself. A study used two different "biosensor" molecules, which were CaM-binding peptides with FRET probes attached, to analyze CaM association with a target peptide (Song, Saucerman, Bossuyt, & Bers, 2008). The advantage to this method is that it allows the activity of CaM to be measured without directly affecting the CaM protein itself. However, this fluorescent technique cannot be expanded to include other experimental designs—a new biosensor molecule would need to be generated for each peptide to be tested. Since CaM regulates hundreds of enzymes, finding a suitable FRET-labeled peptide fragment of each enzyme represents several lifetimes of work.

Thus, a major problem with FRET-based approaches to CaM research stems from the small size of CaM. CaM is only 149 amino acids long, while FRET probes are large aromatic-rich molecules with hefty molecular weights. Attaching two FRET probes to CaM might overwhelm the protein's ability to move through space and carry out its normal functions. The observation technique probably interferes with the process being observed; there are probably better solutions. In an effort to generate a CaM protein that has relevance in both biochemical and physiological model systems, extrinsic fluorescence should be used, but the size of the fluorophore must be minimized so that the the natural behavior of CaM is not affected. Furthermore, due to the promiscuity of CaM binding, the most effective model system will place the fluorophore on CaM itself, and not on a specifically-designed fluorescent CaM target.

Small, fluorescent molecules have been used to tag CaM before. Generally speaking, these molecules are usually designed to covalently link with the unique thiol chemistry specific to cysteine residues. Attaching fluorophores to cysteine residues is advantageous because cysteine is the only amino acid with a thiol group, allowing for the fluorophore to be introduced at very specific attachment points. However, mammalian CaM does not natively express any cysteine residues (see Figure 2). Plant CaM, such as the CaM found in wheat, does contain a cysteine residue. Taking the location of the cysteine residue for granted, a research group tested the effects of fluorescent labeling with four different fluorophores. They concluded that cysteine-bound small fluorophores didn't noticeably affect CaM's ability to activate a target enzyme (Zot, Aden, Samy, & Puett, 1990). This particular research is exciting for my study, because preliminary evidence already exists that suggests that small fluorescent molecules can be introduced into CaM without affecting CaM's behavior.

There is a missing piece at the junction of the FRET modelers and the plant CaM researchers. An opportunity exists for the ideas that plant calmodulin researchers to be used in animal-centric research. All that's missing is cysteine residues in mammalian CaM. If a researcher is able to introduce a cysteine residue into the CaM sequence, such as through site-directed mutagenesis, they would be able to engineer a fluorescent CaM with the fluorophore attached wherever they chose to place the cysteine residue. Finding the best location to introduce a cysteine residue into CaM so that it can be studied via small molecule fluorescence is the crux of this thesis.

Troponin C fluorescent research. Before attempting to design a novel fluorescent calmodulin by introducing a cysteine residue, then attaching an extrinsic fluorophore, it makes sense to see what strategies have worked in proteins similar to CaM. One similar protein is troponin C, another calcium-binding protein of the EF-hand family. Remember, calmodulin is a calcium sensor that is expressed in every animal cell, and is used to regulate hundreds of different enzymes. In contrast, troponin C is a calcium sensor that is specific to striated muscle tissue. Striated muscles include cardiac and skeletal muscles. Just like calmodulin, troponin C binds to calcium, undergoes a conformational change, and activates a target protein. Instead of activating hundreds of different proteins, however, troponin C is only a regulator of troponin I. When troponin C binds to calcium, it causes troponin I to no longer inhibit the contraction of striated muscle (Kobayashi, Jin, & de Tombe, 2008).

Despite their differences in binding partners, troponin C and calmodulin are strikingly similar in their amino acid sequence. Figure 3 is an alignment of the two proteins' primary structure, which reveals that the proteins have 65% sequence identity. Seeing that the two proteins are very similar "cousins" of one another, one basis for the design of a fluorescent

calmodulin would be to use fluorescent constructs that have worked in troponin C. Specifically, it has been shown that extrinsic fluorescence can be engineered into troponin C, whereby its interaction with troponin I can then be observed. Unlike CaM, cardiac troponin C natively expresses two cysteine residues. But, those cysteine residues can be converted to serines via site-directed mutagenesis. Then, as discussed earlier, a cysteine can be introduced into the protein wherever desired, such as in the place of a threonine or other serine residue. A troponin C with a threonine (T) to cysteine (C) mutation at the 53rd residue (T53C) has been characterized previously as an accurate reporter of troponin C behavior (Davis, et al., 2007). Thus, when designing a fluorescent calmodulin, this previous success in engineering a fluorescent troponin C serves as an excellent point of reference.

Target Peptides. Any fluorescent calmodulin that we hope to use in the presence of other enzymes must be tested to work in a variety of different systems. Calmodulin binds to entirely too many proteins to focus on any one protein as a representative for the whole population. As such, four important enzymes regulated by CaM, chosen somewhat arbitrarily based on their current popularity in a variety of fields of research, will be used as tests of the versatility of fluorescent CaMs generated for this thesis. The four CaM-regulated enzymes to be included in this study are myosin light chain kinase (MLCK), neural nitric oxide synthase (nNOS), calmodulin-dependent kinase II (CKII), and calcineurin (CaN).

Myosin Light Chain Kinase. In humans, there are three forms of MLCK, one for each of the three types of muscle tissue. The most important form of MLCK is expressed in smooth muscle (smMLCK). Since there is no troponin complex in smooth muscle, calcium-dependent contraction is regulated by the CaM-smMLCK complex rather than the troponin C-troponin I complex. In smooth muscle, phosphorylation of the myosin light chain by smMLCK enables

muscular contraction. In contrast, the MLCK expressed in cardiac and skeletal muscle does not directly allow muscular contraction—troponin C does. However, MLCK activity in cardiac muscle has been identified as an important player in hypertrophic cardiomyopathy (Takashima, 2009). The combination of MLCK's dominant role in the contraction of smooth muscle—which regulates arterial tone and thus peripheral resistance to blood flow—with MLCK's subtle role in the regulation of cardiac myosin activation, make it an ideal target for cardiac research.

Current MLCK research supports the claim that fluorescent research is a segmented field of study. A research group interested in activation levels of the enzyme chose to place two fluorescent markers that flank the CaM-binding region of the MLCK enzyme. The study was interested in following the activation of MLCK, as reported by this two-pronged fluorescent probe that senses calmodulin binding, compared to the activation of the myosin regulatory light chain (Mizuno, et al., 2008). Again, we're confronted with a trend where researchers choose to generate their own fluorescent system for a specific enzyme. And, since CaM regulates hundreds of enzymes, potentially hundreds of different fluorescent enzymes would need to be generated to measure how CaM interacts with each of those targets. Alternatively, an opportunity exists to attempt to engineer one (or a few) fluorescent calmodulin constructs, which could be used across the entire spectrum of CaM-dependent enzyme research. Having a well characterized fluorescent CaM standard would help unify different segments of CaM research, rather than forcing each research group to come up with a fluorescent model that suits their specific interests.

Nitric Oxide Synthase. Another CaM-regulated enzyme is Nitric Oxide Synthase (NOS). Unlike MLCK, which acts as a kinase, NOS is a synthase—and as the name suggests, NOS synthesizes nitric oxide (NO). The action of NO is tissue specific. As it pertains to

cardiovascular physiology, NO is a potent vasodilator. The action of NO on endothelial cells is a hotbed for research involving hypertension, atherosclerosis, arteriostenosis, et cetera (Shaul, 2002). By understanding the mechanisms that regulate NOS activity, a cardiac researcher has the opportunity to tap into the natural therapeutic power of NO as an antihypertensive agent.

A recent study published in *Biochemistry* pertains closely to the goals of this thesis. In the study, a FRET-based system was used to analyze how calmodulin changes its conformation as it binds to different NOS isozymes. The most accepted current construct for using FRET on calmodulin is a two-probe system involving the 34th and 110th residue, both of which are threonines. Conversion of those threonines to cysteines, and subsequent attachment of two fluorophores, allows researchers to elucidate how the CaM protein moves in space as it attaches to a target—in this case NOS. The power of this FRET-based approach was validated when it was used to predict correctly the conformation of CaM bound to the edema factor of adenyl cyclase, and that prediction was later confirmed by x-ray analysis (Spratt, et al., 2008). In short, FRET systems are proven effective tools for examining the *location* and *conformation* of CaM molecules.

What's less clearly understood, however, is how these FRET systems affect the *kinetics* or *regulatory behavior* of small proteins such as CaM. The 2008 Spratt study referenced earlier went to great lengths to make sure that their FRET system was accurately able to report quantitatively how the CaM molecule was moving through space as it binds to NOS. This data is absolutely vital for a qualitative understanding of how CaM interacts with targets. However, one shortcoming of this FRET system stems from the dynamic nature of living systems—especially cardiac physiology. Between every heart beat, the cardiac myocyte attempts to sequester intracellular calcium, when suddenly a rush of new calcium enters the myocyte, inducing a new

contraction, and starting the sequestration process over again. With calcium concentrations in a constant flux, the speed at which calcium-sensitive proteins—CaM, for example—gain and lose calcium is pivotal. Thus, in the quest for a fluorescent calmodulin, the kinetics of that fluorescent construct are paramount. Any fluorescent CaM that you want to use in a system relevant to living systems must be able to closely mimic the kinetic behavior of endogenous proteins.

Calcineurin. The third CaM-binding protein to be included in this thesis is calcineurin (CaN). Unlike MLCK, which is a kinase, CaN is a phosphatase. By removing a phosphoryl group on nuclear factor of activated T cells (NFAT)—a transcription factor—calcineurin is able to regulate gene transcription indirectly. The role of calcineurin in immunology is much better understood than its role in cardiac physiology. When a T cell is stimulated to differentiate into a helper T cell, a signal transduction pathway carries that differentiation message from a receptor on the cell surface to the nucleus. Calcineurin is a major checkpoint in that pathway, and only through CaM-dependent activation of CaN can that T cell differentiate to become whatever type of T cell it needs to be. Immunosuppressant drugs that suppress CaN activity, such as cyclosporin, have been around for decades (Liu, 1993).

About a decade ago, the Molkentin lab in Cincinnati made a remarkable and simple discovery about CaN. They found that if you predisposed a mouse to cardiac hypertrophy, but blocked the action of CaN by cyclosporin, that mouse would not develop cardiac hypertrophy (Sussman, et al., 1998). Subsequent analysis, however, would demonstrate that cardiac hypertrophy could not be so easily prevented in humans with cyclosporin therapy, due to such boundaries as the nephrotoxicity of cyclosporin (Leinwand, 2001). What was made clear,

however, was that some facet of cardiac hypertrophy was under regulation by the CaN-NFAT pathway, and should be considered a target for cardiac research.

Recent research in the field has been targeted at elucidating the exact role of CaN in cardiac physiology. Cyclosporin therapies are frequently used in tandem with antihypertensive drugs such as calcium channel blockers or ACE inhibitors. Both the immunosuppressant and antihypertensive drugs have the effect of CaN suppression, which has led researchers to investigate how the widespread silencing of CaN activity affects cardiac health. Tests so far have been fairly inconclusive (Zhang, Zhu, & Zhang, 2009). Furthermore, it is also of special interest to see how the constantly changing calcium concentration in the heart affects CaN activity in comparison to the relatively static calcium environment of other cell types (Houser & Molkenin, 2008). By the same vein, this thesis is interested in embracing the calcium fluctuations of the heart as a pivotal component of cardiac research.

Calmodulin-dependent kinase II. The fourth and final CaM-regulated enzyme to be used for this thesis is CKII. CKII is a kinase that doesn't just phosphorylate one specific target, it has an abundance of possible targets, just like CaM does. About a decade ago, it was observed that CKII is overexpressed in late-stage cardiac disease (Hoch, Meyer, Hetzer, Krause, & Karczewski, 1999). Since then, the field of CKII research has grown tremendously, with different researchers finding different pathways regulated by CKII to focus on. The number of CKII articles published between 2001 and 2006 doubles that of the previous five years (Maier, 2009).

CKII is a particularly important player in cardiac physiology because of its role in the regulation of different calcium handling processes. CKII phosphorylates ryanodine receptors, phospholamban, and L-type calcium channels, all of which play pivotal roles in the management

of intracellular calcium levels and excitation-contraction coupling (Maier & Bers, 2002). It's particularly interesting that CKII regulates a great number of calcium handling pathways, and CKII is also itself regulated by calcium levels.

Objectives

As evidenced by the litany of different techniques being employed to follow CaM behavior using fluorescence, CaM research is an unnecessarily segregated field of study. Researchers develop new fluorescent systems each time they want to answer a specific question, a trend that leads to extra expenses and perhaps overspecialized model systems. Despite the popularity of fluorescence as a tool, no one is taking a moment to develop the best tool possible for the job, and sharing that tool with others. Most fluorescent techniques are either assumed to work, or are too highly specialized for one research question to be at all beneficial to other researchers. As such, it has become apparent that CaM researchers would stand to benefit from someone taking the time to engineer a fluorescent CaM that encapsulates as many possible segments of the CaM research field with one fluorescence approach. A fluorescent calmodulin that has diverse utility would give CaM researchers a common tool that could facilitate crosstalk between different segments of study, as well as different strata of complexity within any one segment of study.

However, it would also be naïve to assume that any one panacea exists to completely unify CaM researchers. FRET approaches are uniquely powerful tools for measuring spatial arrangements, and I imagine that T34C/T110C dual-probe FRET systems will continue to dominate as the standard for modeling CaM movements. Furthermore, biophysicists that model the behavior of single CaM molecules will likely be completely disinterested in a CaM that has a prosthetic fluorophore attached; they can get along just fine relying on intrinsic fluorescence. In

other cases, a fluorescently labeled CaM protein won't give the type of information a biochemist may need if he's studying a CaM target protein such as MLCK. The niche that needs filled is somewhere between all these strategies—there is a need for a fluorescent CaM that is sensitive to CaM's ability to bind calcium *and* CaM's ability to bind with target proteins. Researchers, whether they be interested in NOS, MLCK, or otherwise, would benefit from being able to turn to one fluorescent CaM specifically engineered to behave like WT CaM. They could employ this protein to observe how CaM regulates their enzyme of interest, and be confident that this fluorescent CaM is a reliable representation of the behavior of the wild type protein. Eventually, hundreds of different segments of study could potentially benefit from the development and characterization such a calmodulin.

Since this fluorescent calmodulin is being developed within the walls of a cardiac physiology lab, the most immediate goal is to design and test a fluorescent calmodulin with a smattering of different enzymes important in cardiac physiology. Specifically, the fluorescent calmodulin will be studied in the presence of peptide fragments of the four enzymes discussed earlier in this thesis (MLCK, NOS, CKII, CaN). The best fluorescent calmodulin will be the protein that behaves most similarly to the wild type protein in the presence of the peptides.

The kinetic behavior of the engineered CaMs will be measured, fit mathematically, and compared to one another via statistical analysis. The kinetic behavior of a fluorescent CaM must not be significantly different from the wild type in order to be considered successful. The simplest and ideal result would be one fluorescent CaM that behaves statistically similar to the WT in the presence of all four peptides. Other results—where a fluorescent CaM behaves like the WT in the presence of one peptide but not others—will have to be interpreted more carefully.

Chapter II

Methodology

The basic techniques to be used to generate fluorescent CaM proteins are, for the most part, well understood. An overview of the methodological strategy follows, and subsequent subsections detail each individual step. Please note that specific buffer composition and other cumbersome language have been omitted from the text of this thesis for the sake of readability, with a few exceptions. A copy of the protocols used for this thesis have been included at the end of the report should any questions arise.

The first step to creating a calmodulin mutant is to choose which genetic mutations should be generated. RasWin was employed as a viewer of the crystal structures for calmodulin-peptide complexes, such as CaM-RS20 complex and CaM-NOS complex. Threonine residues in the N-domain that appeared to orient themselves away from the interior of the protein were identified as candidates for mutation (T5, T34). A third mutation (T44C) was made based on a successful fluorescent construct from troponin C. Mutations were made to a pET3d plasmid containing the rat CaM gene via primer-based PCR and site-directed mutagenesis using the Stratagene QuikChange site-directed mutagenesis kit. After confirming the mutations by sequence analysis, mutant plasmids were transformed into BL21(DE3)pLysS *Escherichia Coli* cells and induced to express the introduced CaM gene. Cells containing the mutant CaM gene were then lysed by sonication, and the insoluble cellular elements were removed by centrifugation. Subsequent treatment with ammonium sulfate precipitated out a few water-soluble proteins in the mixture, which were discarded as waste. The remaining solution, which contained CaM, was purified using column chromatography on a phenylsepharose column, yielding highly purified calmodulin.

A fraction of the purified calmodulin was reacted with [2 - (4' - (iodoacetamido) anilino) naphthalene -6 - sulfonic acid] (IAANS). The concentration of the labeled CaM protein was determined by spectrophotometry. In terms of data collection, the calcium dissociation rates of the CaM protein were determined using an Applied Photophysics Ltd. model SX.18 MV stopped-flow instrument with a dead time of 1.4 ms at 20 °C. Since the WT protein could not be labeled with IAANS, its calcium dissociation rate was measured with Quin-2, a fluorescent calcium chelator. A program that uses the Levenberg-Marquardt algorithm was used to fit the data to a double exponential curve. Calcium dissociation rates were compared to one another via one-way ANOVA, with p values <0.05 taken as significant.

CaM mutant design and creation. Crystal structures for the four CaM complexes to be studied were obtained from the Protein Data Bank, which can be found at rscb.org. The four models specifically used were 1QTX (RS20), 2R28 (CaN), 1MXE (CKII), and 1NIW (NOS) [See Figure 4]. The threonine residues in the N-domain of CaM were examined in each of the four crystal structures. T5 and T34 were chosen as potential candidates for mutation based on their R group's orientation away from the interior of the complex. It seems logical that if the probe is engineered to stick out away from the protein that the core functions of the protein will not be sterically inhibited by the presence of the fluorophore. The T44 residue was identified as a candidate for mutation not based on crystallographic analysis, but based on the success of a homologous mutation that provided a successful fluorescent construct in troponin C. Since CaM and troponin C share 65% of their sequence, it seems worthwhile to attempt to mimic the success of the T53C-IAANS fluorescent construct of troponin C (Davis, et al., 2007).

The rat CaM gene, whose primary structure is completely identical to human CaM, was already available in my lab from previous work with CaM systems. The pET3d plasmid that

contains the CaM gene also codes for ampicillin resistance, has a *lac* operon, as well as other functionally important properties. Mutations to the CaM gene on the plasmid were accomplished using primer-based PCR, in which the plasmid is copied repeatedly, using a primer containing a replacement sequence that contains a desired mutation, such as T5C. The PCR reaction preferentially copies the mutant protein DNA, due to its tremendous excess in the solution. Leftover parent DNA was removed from solution by cleavage with the *DPNI* enzyme that digests methylated DNA. The result is a concentrated stock of CaM plasmid with the mutant CaM in the place of WT CaM. The plasmid was repaired when it is transformed into XL1-Blue *E. Coli* cells, whereby the cell machinery ligates the synthetic plasmid from the PCR reaction back into circular plasmid DNA. This circular plasmid DNA was then extracted from the XL1-Blue cells using a Quiagen Mini-Prep DNA extraction kit. The success of the mutation was verified by sequence analysis, which for this research was conducted by the Plant Microbe Genomics Facility at Ohio State.

Protein Expression and Purification. Once the sequence of our mutant plasmids was confirmed by sequence analysis, the mutant plasmids were transfected into BL21(DE3)pLysS *E. coli* cells. The BL21(DE3)pLysS strain of *E. coli* is designed to facilitate expression of our mutant DNA contained in the mutant plasmid. BL21(DE3)pLysS cells were brought up to a 1L culture in LB medium, and the cells were made to begin expressing CaM by addition of a protein called IPTG. IPTG is a transcription factor that turns on the *lac* operon, which is the upstream promoter of the CaM gene in this plasmid system. Induction by IPTG was allowed to progress for three hours, after which the cells were extracted from the media via centrifugation. The cells were resuspended in a solution of protease inhibitors, and the cell membranes were lysed via sonication. Insoluble organelles and cytosolic elements were removed via centrifugation. The

resultant solution is a concoction of numerous soluble proteins. Since CaM is resistant to precipitation by ammonium sulfate, a fraction of the protein contamination in the solution was precipitated out by addition of ammonium sulfate. Precipitated proteins were again removed by centrifugation. The leftover solution was then given a large dose of calcium, and then loaded onto a phenylsepharose column. The calcium serves to saturate calmodulin, which exposes very hydrophobic regions within the CaM molecule, allowing it to stick to the phenylsepharose column. After washing the column, CaM is eluted with the addition of EGTA, which chelates calcium away from CaM, hiding CaM's hydrophobic regions, and causing CaM to elute from the hydrophobic column.

Protein labeling. Approximately half of the purified CaM was labeled with the chosen fluorophore—IAANS. IAANS reacts selectively to the thiol group on the cysteine that was engineered into the CaM sequence. CaM was treated with dithiothreitol (DTT) to reduce its thiol group to ensure proper reactivity with the IAANS probe. DTT was then dialyzed out of the protein solution, and urea was dialyzed into the solution to denature the CaM protein. IAANS was dissolved in dimethylformamide (DMF) and added to the CaM protein in five-fold excess to ensure maximal contact between the protein and the fluorophore. The reaction proceeded for two hours at room temperature. Excess fluorophore and urea is removed from the protein solution by extensive dialysis. The result is a solution of protein labeled at the mutant cysteine residue with the IAANS probe.

Kinetic Characterization of Calmodulin. The calcium dissociation rates (K_{off}) of the three Calmodulin-IAANS fluorescent mutants were determined by stopped-flow spectrophotometry. The exact apparatus used was an Applied Photophysics Ltd. model SX.18 MV stopped-flow instrument with a dead time of 1.4 ms. This experimental design relies on two

different methods for measuring fluorescence. For our experimental group—the engineered fluorescent calmodulins—IAANS fluorescence was measured by exciting the sample at 330 nm and emission was collected with a photomultiplier tube fitted with a 420-470 nm filter. For the control experiments—using WT CaM—Quin-2 was used as the source of fluorescence. Quin-2 was excited at 330 nm as well, and its emission was filtered through a 510 nm broad band pass interference filter. A program that uses the Levenberg-Marquardt algorithm was used to fit the data to a double exponential curve, which yields a rate constant for the calcium dissociation event we were using. The buffer composition for all kinetic experiments is as follows: 10 mM MOPS, 150 mM KCl, 3 mM Mg^{2+} , 1 mM DTT, pH7.0. The MOPS is a pH buffering reagent, the KCl, Mg^{2+} , and H^+ concentration mimic intracellular ion concentrations, and DTT is used as a reducing agent to protect against protein oxidation. All kinetic experiments were conducted at 20 °C.

Experimental Design

One of the most important aspects of this study is establishing an experimental design that allows us to answer a few basic questions. For one, how can we be sure that our designed fluorescent constructs behave statistically similar to the WT protein? And, secondly, can we determine where in the manufacturing process any proteins that don't behave like the WT protein lose their WT-like characteristics? Do they become different after the genetic change we are making within the protein (the T to C mutation) or after we covalently link them to the fluorophore?

There are multiple ways for us to answer whether or not the engineered proteins behave like the WT protein. Calmodulin has two basic functions. One, it binds to and dissociates from calcium ions. Secondly, CaM binds to and dissociates from target proteins. Both phenomena

can be tracked via the fluorophore we've introduced into the protein. However, designing a control experiment for calcium binding is much more clear-cut than designing a control experiment for protein-protein binding. Ultimately, we need to measure how well our engineered calmodulin mutants behave compared to the WT protein.

Furthermore, there are multiple methods to examine the behavior of a protein. Two basic approaches to a calcium-binding protein such as calmodulin are steady-state experiments and kinetic experiments. Steady-state experiments are usually titrations, in which calcium or a target protein is titrated into a solution of calmodulin, and fluorescence is used to determine the concentration at which calmodulin is able to bind to either calcium or the target protein. Alternatively, kinetic experiments determine the rate at which these binding or dissociation events occur. So, instead of determining the effective concentration at which a target protein is able to bind with CaM, a kinetic experiment tells you how fast that reaction occurs. Both are important pictures of CaM function, and good indicators to use when determining whether or not a mutant protein behaves like the WT variety.

When working with an unknown, new fluorescent construct, it's beneficial to use one technique to build intuition about the other technique. That is, the fluorescent activity during kinetic experiments should relate to the fluorescent activity of steady-state experiments. For example, when a kinetic experiment is conducted in which CaM loses calcium over time, and also loses fluorescence over time, one could reason that high fluorescence correlates with large amounts of calcium bound to the protein. Thus, when that same fluorescent protein is used in a steady state experiment, one would expect for the fluorescence of the CaM solution to increase as calcium is titrated into the reaction mixture.

This, unfortunately, is an oversimplification of the reality of CaM behavior. CaM has four calcium binding loops, two in each domain of the protein. Past experiments have demonstrated that the C and N-terminal halves of the protein have different calcium sensitivities, and thus different kinetic behavior (VanScyoc, et al., 2002). In addition to this, the two domains of calmodulin also have different effects on the fluorescent behavior of the protein. Preliminary experiments revealed the following trends regarding CaM labeled with IAANS in the N-domain.

- i. The N-terminal domain of calmodulin has a low sensitivity to calcium when compared to the C-terminal domain. This makes the N-terminal domain lose calcium much faster than the C domain. The N-terminal domain has low fluorescence when calcium-bound, and high fluorescence when calcium-free.
- ii. The C-terminal domain has higher calcium affinity than the N-terminal domain. C-terminal kinetics are much slower than N-terminal kinetics. C-terminal fluorescence is high when it is bound to calcium, and low when it is not.

The result of these two observations makes titrations very difficult. As calcium is added to any of our three engineered CaM proteins, the fluorescence signal at first increases as the C-terminal domain grabs calcium. Further calcium additions cause the fluorescence signal to decrease, which is a result of the N terminal domain grabbing calcium. Biphasic titrations such as this are very difficult to characterize, and efforts to study the steady-state behavior of the IAANS labeled CaM mutants are ongoing. Thus, for the constraints of this thesis, the more easily interpreted kinetic data are presented as the representative for CaM function.

This, however, plays well into the context of the research. We are most interested in how we can use these designed fluorescent calmodulins to gain a better understanding of how calmodulin regulates a spectrum of different pathways that relate to cardiac function. We also

know that calcium concentrations of cardiac myocytes are in constant flux. With the constant calcium transients of cardiac tissues, the most important characteristic for these engineered fluorescent CaM proteins to retain is their kinetic properties.

Another point we need to consider, before we delve into the specifics of what experiments will be performed, is the duality between the N and C terminal sides of the protein, their role in regulation of cardiac systems, and what that means for engineered fluorescence. It has been shown that the concentration of CaM freely floating around in the cytosol of cardiac myocytes is on the order of 50-75 nM. This, surprisingly, is only 1% of the total CaM in a given cardiac myocyte. This research suggests that CaM is almost always active and able to bind to target proteins (Wu & Bers, 2007). There are several reasons why CaM could be almost always bound to target proteins. For one, the classical model that CaM must first bind calcium and then bind a target protein could be flawed. Proteins with high affinity for CaM are probably binding to the CaM protein even at resting calcium concentrations. Another implication of this research is that the C domain—the more sensitive domain to calcium—could be bound with calcium even at resting calcium concentrations, which partially activates CaM and allows it to bind with targets. Calcium fluctuations, from that point, would be targeted at the N-domain of the protein, which would bind with calcium and activate whatever protein CaM was already bound to.

While these non-classical mechanisms of CaM activation have yet to be fully understood, what is clear is that the N-terminal domain of CaM has a sensitivity to calcium that is more likely to be directly affected by rising and falling calcium transients in a beating myocyte. As such, it is a greater priority to understand what is happening in the N-terminal domain of CaM more so than the C-terminal domain, since the sensitivity of the N-terminal domain seems to have a more direct role in the regulation of CaM function. This line of reasoning is the basis for why the

three fluorescent mutants in this study were located in the N-terminal domain, and why our experimental design will also focus on the N-terminal domain kinetics.

Selection of fluorophore. The engineering strategy being employed in this study—where one prosthetic group is being attached to a cysteine residue—limits which fluorophores can be used. The Invitrogen Molecular Probes Handbook details a family of different cysteine-reactive environmentally sensitive probes that could be used in this experiment. Each probe has a molecular weight on par with a tryptophan residue. Preliminary research for this study identified that IAANS gave the best signal to noise ratio of the fluorophores available in our lab, and so it was used as the fluorophore of choice when labeling the three mutation candidates. Consult figure 5 for the structure of IAANS.

Experimental Protocol. Finally, the experiments themselves. The experimental group—T5C IAANS, T34C IAANS, and T44C IAANS—will all be analyzed in the stopped-flow spectrophotometer. Figure 6 is a simple schematic diagram of a stopped-flow apparatus. Measurements of calcium dissociation rates will be achieved by observation of the rapid mixing of two solutions. The first solution will contain a buffer, an IAANS-labeled CaM protein, a peptide fragment of one of the four proteins we've chosen (NOS, CaN, CKII, RS20), and calcium. Imagine this syringe containing essentially any of the four complexes depicted in figure 4. The second syringe will simply contain the same buffer and a tremendous excess of EGTA, a calcium chelator. EGTA is able to chelate calcium instantaneously as CaM dissociates from it, and since an equilibrium exists between calcium-bound CaM and calcium-free CaM, EGTA sequesters all calcium that CaM dissociates from for even the slightest amount of time.

The kinetic characteristics of the WT CaM protein, our control, will be measured using a fluorescent cousin of EGTA called Quin-2. Quin-2 has widely been used as a fluorescent stand-

in for EGTA (Tikunova & Davis, 2004). Just like the previous experiment, WT calcium-bound CaM bound to a peptide will be mixed with a calcium chelator. In this control experiment, however, it is not the CaM protein itself that is fluorescing, but the Quin-2 chelator. The rate of Quin-2 fluorescence change is equal to the rate at which CaM loses calcium. This Quin-2 technique will also be employed to analyze whether or not the genetic mutation from threonine to cysteine has any effect on calcium dissociation rates, for the stock of proteins that have undergone mutation but have not been labeled with IAANS.

Chapter III

Results

The results for our kinetic characterization of the calmodulin mutants are displayed in figures 7 through 10. The difference between these figures is the peptide used with CaM. The colored traces represent the time course in which CaM transitioned from its initial fluorescence before mixing with EGTA or Quin-2, to the point where the experimental solution reached its maximal fluorescence. This fluorescent plateau is interpreted to represent the point at which all calcium had been lost from the N-terminal domain of CaM.

A statistical analysis of these four figures can be found in tables 1 through 4. The n values represent how many total traces were averaged to find the average calcium dissociation rate K_{off} . A one-way ANOVA test was employed to test for significant differences from the WT. For the purposes of this study, any fluorescent construct that behaves significantly different from the WT is unsuccessful, at least in that particular peptide system.

According to the statistical analysis, the T44C construct was significantly different ($p < 0.05$) from the WT in each of the four peptide systems used. The T5C construct behaved

significantly different from the WT only in the CKII system. The T34C construct behaved significantly different from the WT in the CaN system.

A qualitative analysis of the difference between the T5C and T44C CaM is available in figure 11. Both of these traces compare three steps along the process. First, a WT Quin-2 trace is shown. The next trace is a representative of the protein after the T to C genetic mutation has been made, but before the protein has been labeled with IAANS. The last trace is then the calcium dissociation rate of the protein with IAANS. This data suggests that the genetic mutation and the fluorescent labeling of T5C IAANS affect the calcium dissociation kinetics in equal but opposite ways. However, the genetic mutation and labeling of T44C CaM both sum to create a much faster CaM overall in terms of N-terminal calcium kinetics.

Conclusions

Although nothing in the statistical analysis here allows me to say what is statistically similar to the WT probe, what can be determined is which probes were most consistently *not significantly different*. Both T5C and T34C showed fairly equal success, both being found to be not significantly different from the WT in three of the four peptides used. However, the T5C fluorescent construct demonstrates a superior signal-to-noise ratio to the T34C fluorescent construct. Specifically, the width of the data traces are noticeably narrower for T5C than T34C. Based on that observation, I would chose to use the T5C probe over the T34C probe in systems where both were shown to not behave significantly different from the wild-type.

What's particularly interesting is that the three fluorescent constructs seemed to deviate most strongly from the WT in terms of average K_{off} in the system with a non-classical CaM-peptide association pattern, the CaN system. As shown in figure 4, CaM binds to CaN in a 2:2 antiparallel arrangement in the best available crystal structures, whereas the other binding

patterns shown in figure 4 resemble the classical arrangement of calmodulin wrapping around a target peptide. It is unclear as to whether this 2:2 binding arrangement is specific to the peptide subunits being employed in this study, or if this 2:2 binding arrangement is preserved in the intact CaN holoenzyme.

To be able to measure these same calcium dissociation rates in the presence of intact holoenzymes would be an exciting next step for this research. These four peptide constructs have served as a proof of principle that CaM calcium dissociation rates can still be reliably measured in the presence of CaM targets. What remains unclear is whether or not the constructs created to work with these peptides will also work with the entire enzyme. It is very possible to imagine that distal domains of the target enzymes might sterically interact with the IAANS fluorophore and that fluorophore will no longer properly report the true calcium dissociation rate of the CaM protein. It is very exciting, however, to imagine the best case scenario—where the T5C or T34C IAANS fluorescent CaM constructs continue to reliably report calcium dissociation rates in the presence of whole enzymes.

However, for the time being, the model system we've generated has shown promise. At the first trial of their reliability, the T5C IAANS and T34C IAANS fluorescent CaM constructs have been shown to reliably report calcium dissociation rates in at least a few peptide systems. In the future, these fluorescent models will be further characterized, and perhaps employed as a common tool for a diverse population of CaM researchers. At the very least, we've taken the first steps to ensure that our CaM fluorescent system, a carefully selected reductionism of the overwhelmingly complex landscape of cardiac research, generates reliable data that we can confidently share with the scientific community.

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Appendix A

Calmodulin Purification Protocol

1. **Transform** the CaM plasmid into BL21 bacteria.
2. **Grow** transformed BL21 bacteria in 1 L flasks of LB media + Amp, until OD₆₀₀ is 0.6-0.8
3. **Add** 0.4 mM IPTG (1 mL of a .4 M stock)
4. **Grow** cells with IPTG for another 3-4 hours
5. **Spin** cells for 15 min @ 8,000 rpm, 10 deg. C
6. **Resuspend** cells in 30mL resuspension buffer for every 1 L of starting volume
 - a. **Resuspension buffer** – 50 mM Tris, 2mM EDTA, 1mM DTT, 1mM PMSF, pH 7.5
7. **Sonicate** cells on ice – Output 6, duty cycle 60%, 6 times for 2 minutes each.
8. **Spin** solution for 30 minutes @ 11,500 rpm and 4 deg. C.
9. **Collect** supernatant
10. **Add slowly** 27g of ammonium sulfate per 100 mL (45% saturation) and let stir for 1 hr. Perform this step while stirring in the cold room.
11. **Spin** solution for 30 minutes @ 11,500 rpm and 4 deg. C (keep supernatant)
12. **Equilibrate** phenylsepharose Cl-4B column (~15 mL resin) with buffer A.
 - a. **Buffer A** – 50 mM Tris, 1mM DTT, 500 μM Ca²⁺, pH 7.5
 - b. Note: See Phenylsepharose Column Prep Protocol if necessary.
13. **Add** 5mM Ca²⁺ to the supernatant from step 11, and load onto column.
14. **Wash** column with buffer A until OD₂₈₀ of flow through falls below 0.03.
15. **Wash** column with buffer B until OD₂₈₀ of flow through falls below 0.02.
 - a. **Buffer B** – 50 mM Tris, 0.5 M NaCl, 500 μM CaCl₂, 1mM DTT, pH 7.5
16. **Elute** with buffer C
 - a. **Buffer C** – 50 mM Tris, 0.15 M NaCl, 5 mM EDTA, 1mM DTT, pH 7.5
17. **Collect** 5 mL fractions (typically comes off between 3rd to 6th fraction)
18. **Measure** OD₂₈₀.
 - a. wtCaM extinction coefficient @280 is 3140
19. **Place** pulled fractions into dialysis
20. **Run** on gel

Appendix B

Protein Labeling with IAANS protocol

1. **Place** proteins to be dialyzed into 4 degree fridge to allow thaw time.
2. **Reduce** proteins in 5mM DTT for a few hours before step 3.
3. **Dialyze** 100 μ M of protein (probably 3-5 mL of fraction with the highest OD) against 2 L **urea buffer** overnight.
 - a. **Urea buffer** – 50 mM Tris, 90 mM KCl, 1 mM EGTA, 6 M Urea, pH 7.5
4. **Check** OD₂₈₀ of dialyzed protein and determine concentration
 - a. CaM Extinction coefficient = 2560 (SCaM 1 is 1280)
5. **Make** 10 mM IAANS in dimethylformamide (DMF) (0.5 mg/ 100 μ L)
 - a. Make a fresh stock big enough for the proteins that need labeled that day.
 - i. The concentration of added label should be 5x the protein conc.
 - ii. Eg. 150 μ M protein needs 750 μ M label
6. **Add** IAANS slowly, dropwise, and shake for 5-8 hours at 4 °C. (parafilm lids)
 - a. Alternatively, shake for 2 hours at RT.
7. **Add** 2 mM DTT to stop the reaction.
8. **Dialyze** overnight against urea buffer.
9. **Dialyze** against 3 changes of 4 L of:
 - a. 10 mM MOPS, 90 mM KCl, pH 7.0

Appendix C

Reaction Mixtures for Stopped Flow Spectrophotometry

IAANS Experiments	
Syringe A	Syringe B
0.5 μM CaM 3 μM peptide 200 μM Ca^{2+} Buffer	10 mM EGTA Buffer
Quin-2 Experiments	
Syringe A	Syringe B
3 μM CaM 20 μM peptide 20 μM Ca^{2+} Buffer	150 μM Quin-2 Buffer

Note: See Figure 6 for a schematic diagram of stopped flow spectrophotometry and how syringe A and B interact.

Table 1

Statistical Analysis of RS20 System

Protein	n	Avg. K_{off} (/s)	<i>p</i>
WT	15	7.8 ± 0.20	
T44C	15	24.4 ± 2.95	0.000
T34C	20	6.5 ± 0.21	0.207*
T5C	20	8.5 ± 0.31	0.561*

*Note: *p > 0.05*

Table 2

Statistical Analysis of CaN System

Protein	n	Avg K_{off} (/s)	<i>p</i>
WT	15	12.0 ± 0.85	
T44C	15	28.6 ± 3.01	0.000
T34C	20	17.9 ± 0.72	0.0158
T5C	20	15.8 ± 1.11	0.0799*

Note: * $p > 0.05$

Table 3

Statistical Analysis of NOS System

Protein	n	Avg. K_{off} (/s)	<i>p</i>
WT	15	19.6 ± 1.85	
T44C	15	44.8 ± 4.56	0.000
T34C	20	26.3 ± 2.61	0.0632*
T5C	20	16.5 ± 1.68	0.233*

Note: * $p > 0.05$

Table 4

Statistical Analysis of CKII System

Protein	n	Avg K_{off} (/s)	<i>p</i>
WT	15	12.6 ± 0.52	
T44C	15	29.0 ± 2.68	0.000
T34C	20	12.0 ± 0.95	0.736*
T5C	20	9.0 ± 0.48	0.0227

Note: * $p > 0.05$

Figure Captions

Figure 1. The Crystal structure of CaM protein represented in ribbon form. Black orbs represent calcium ions (Rupp, Marshak, & Parkin, 1996). The zig-zag portion at the bottom of the diagram represents a tail of the protein that did not resolve clearly in the crystal structure.

Figure 2. The primary structure of calmodulin protein is shown at the top, with mutated residues colored in cyan, yellow, and magenta. The protein structure below that indicates the location of those three residues within the protein three-dimensional structure. The yellow, cyan, and magenta color scheme here is maintained throughout the report. The colors alternate between black and red every ten residues to assist in counting.

Figure 3. Alignment of human calmodulin (hCaM) with cardiac troponin C (cTnC). Residues with >90% consensus are shown in green, and mismatched residues are shown in black and red. 97 of the 149 overlapping residues are high consensus (identical or very functionally similar), indicating that these two proteins are 65% identical.

Figure 4. Comparison of the crystal structures of Calmodulin with each of the four peptides used in this study. The crystal structures used were models 1QTX (RS20), 2R28 (CaN), 1MXE (CKII), and 1NIW (NOS). Each of these models is available from the protein data bank, rscb.org. The CaN complex has so far only been simplified to the 2:2 binding scheme shown in the diagram. In all cases, the peptide is shown in red, and CaM is shown in navy. The additional green protein in the CaN structure is another CaM molecule, arranged in an antiparallel relationship with the navy colored CaM molecule.

Figure 5. Structure of the IAANS probe (Invitrogen 2009).

Figure 6. Schematic Diagram of a stopped-flow apparatus (Lehrer 2004).

Figure 7-10. Real data is shown whenever possible. All traces shown are a representative of the average, rather than an aggregate sum. Fit curves were used where data would obscure other data from view, or when the signal-to-noise ratio of that data made its trends not immediately visible. Most commonly, those fit curves were used to display the data for the T5C and T34C constructs, and the figure legends indicate which curves are fits.

Figure 11. Comparison of the phases of T5C construct generation to the phases of T44C construct generation. Data was collected using the RS-20 peptide system, such as that used for the experiment shown in figure 7.

Figure 1

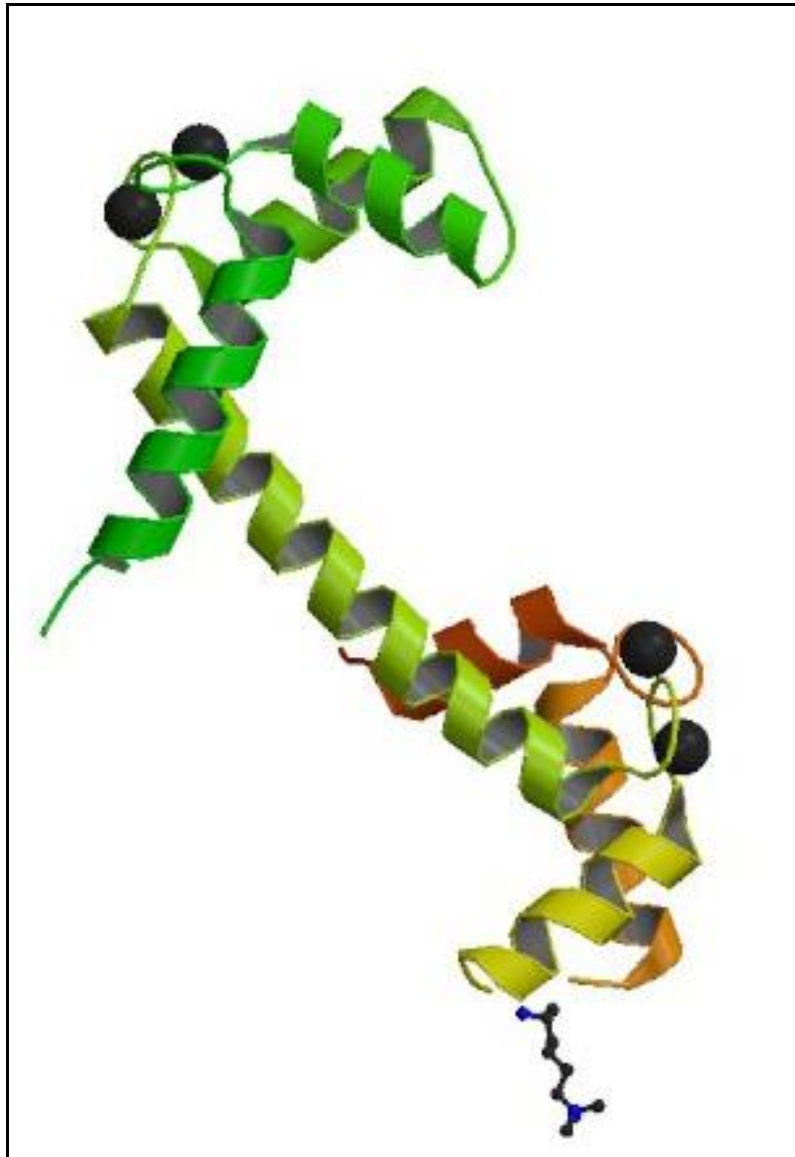


Figure 2

MADQL**T**EEQIAEFKEAFSLF**DKDGDGTITT**KELG**T**VMRSLGQNP**T**EAELO
DMINEVDADG**NGTIDFPEFL**TMMARKMKD**TDSEEEIREAF**RVFDKDGNGY
ISAAELRHVMTNLGEKLTDE**EVDEMIREAD**IDGDGQVNY**EEFVQM**MTAK

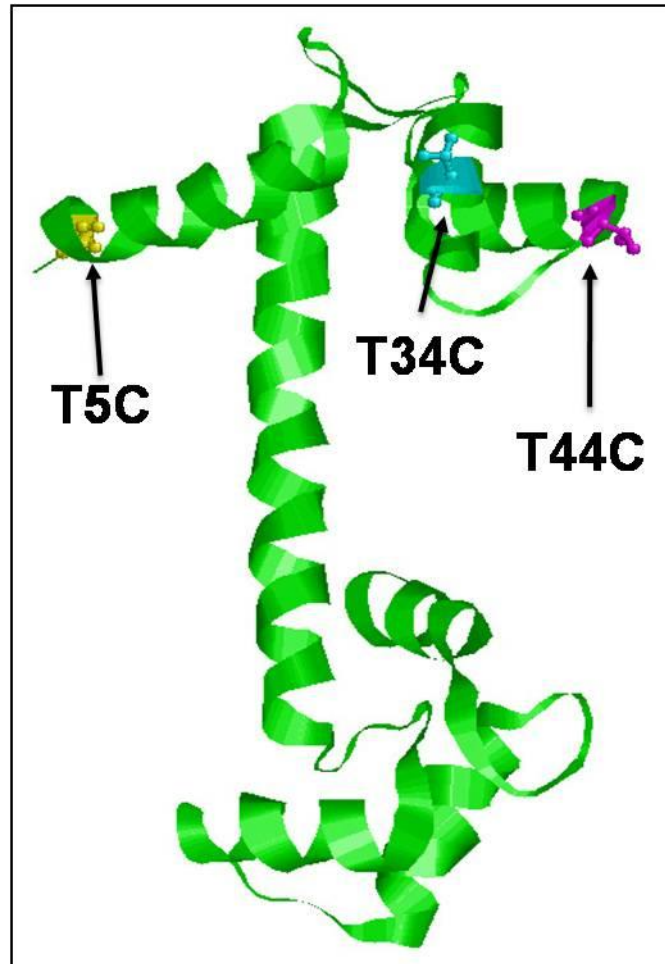


Figure 3

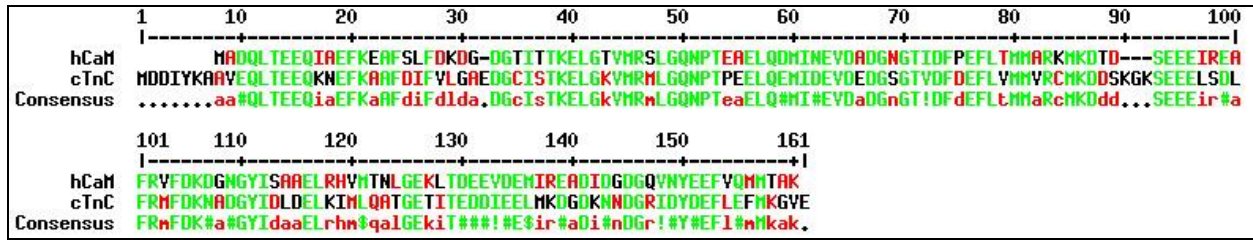


Figure 4

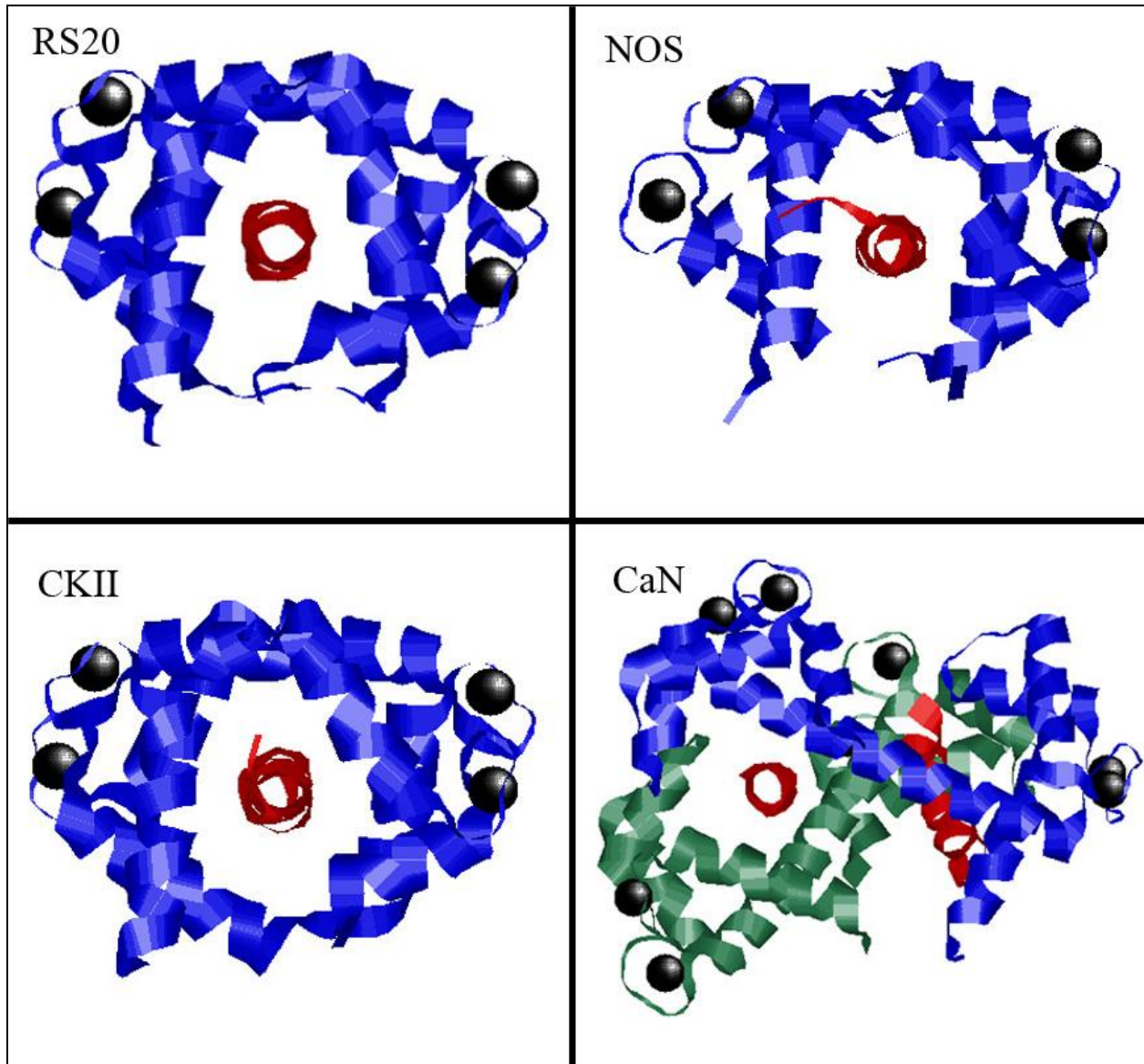


Figure 5

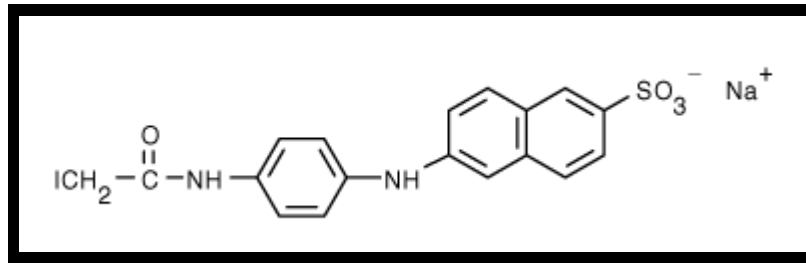


Figure 6

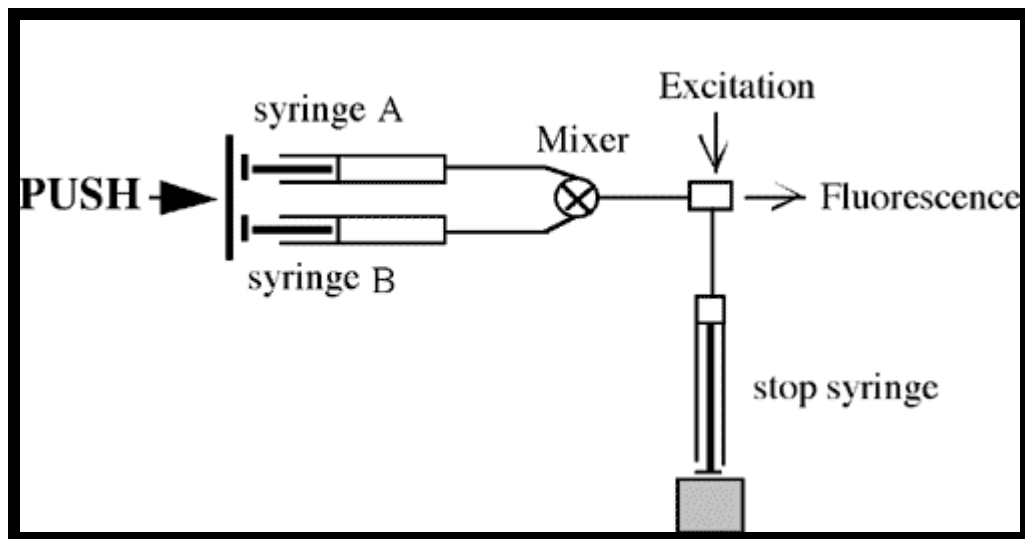


Figure 7

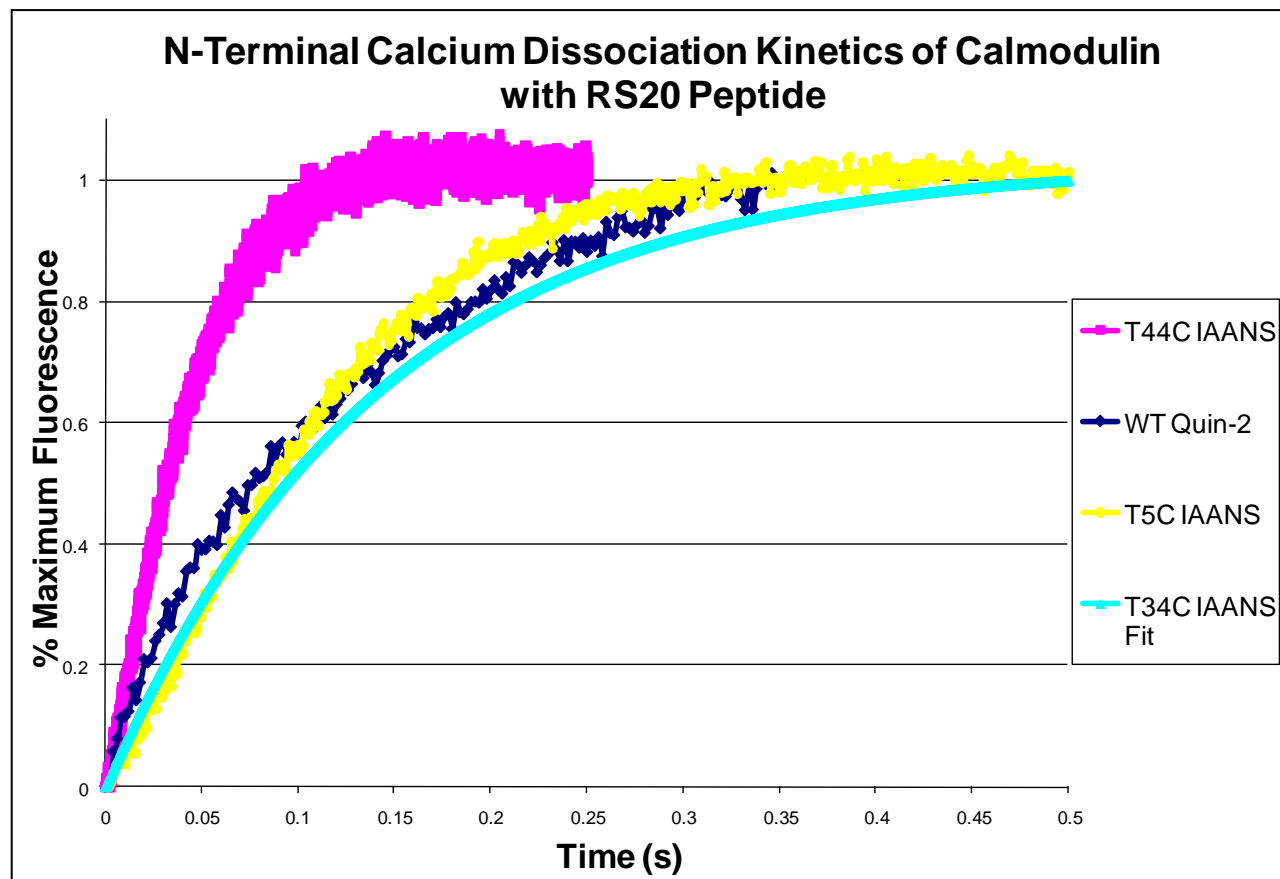


Figure 8

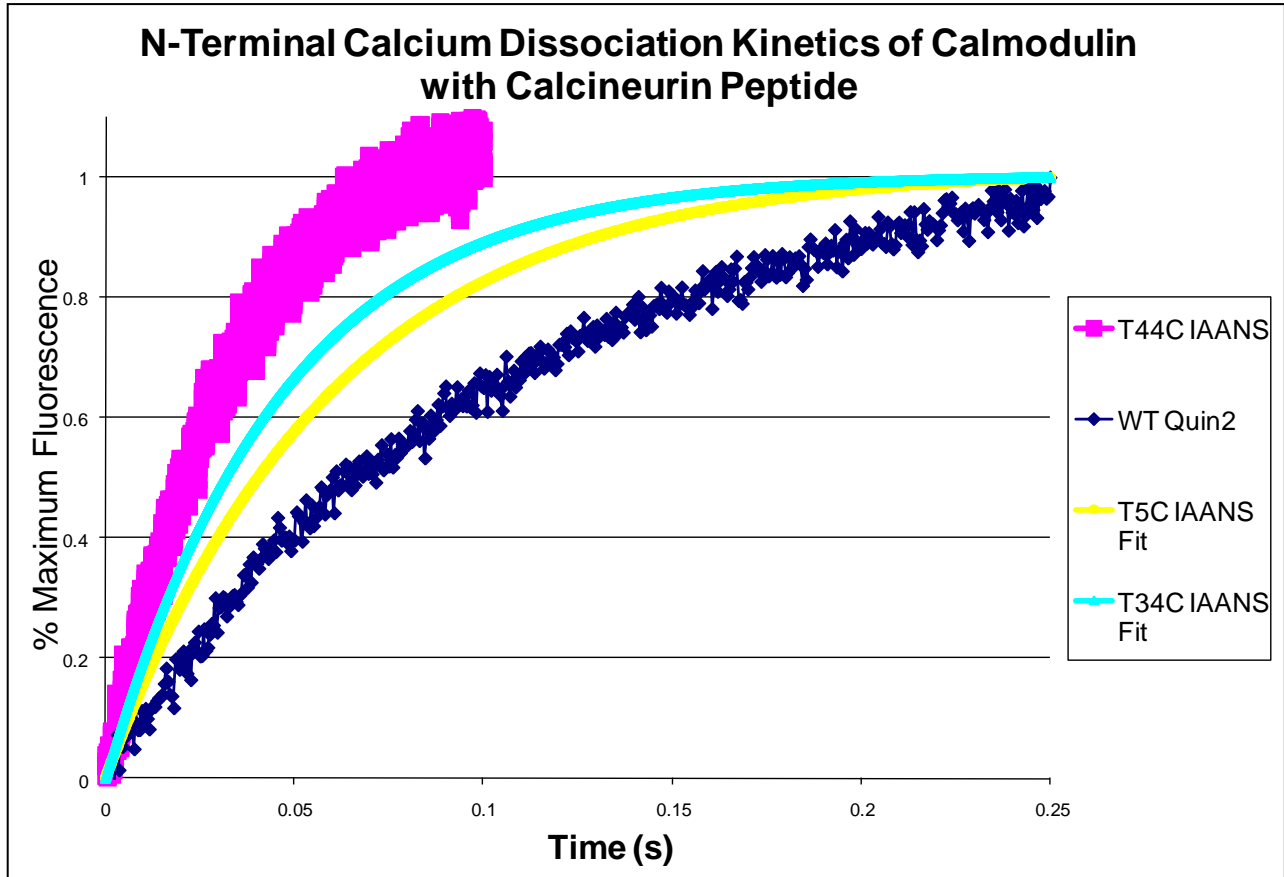


Figure 9

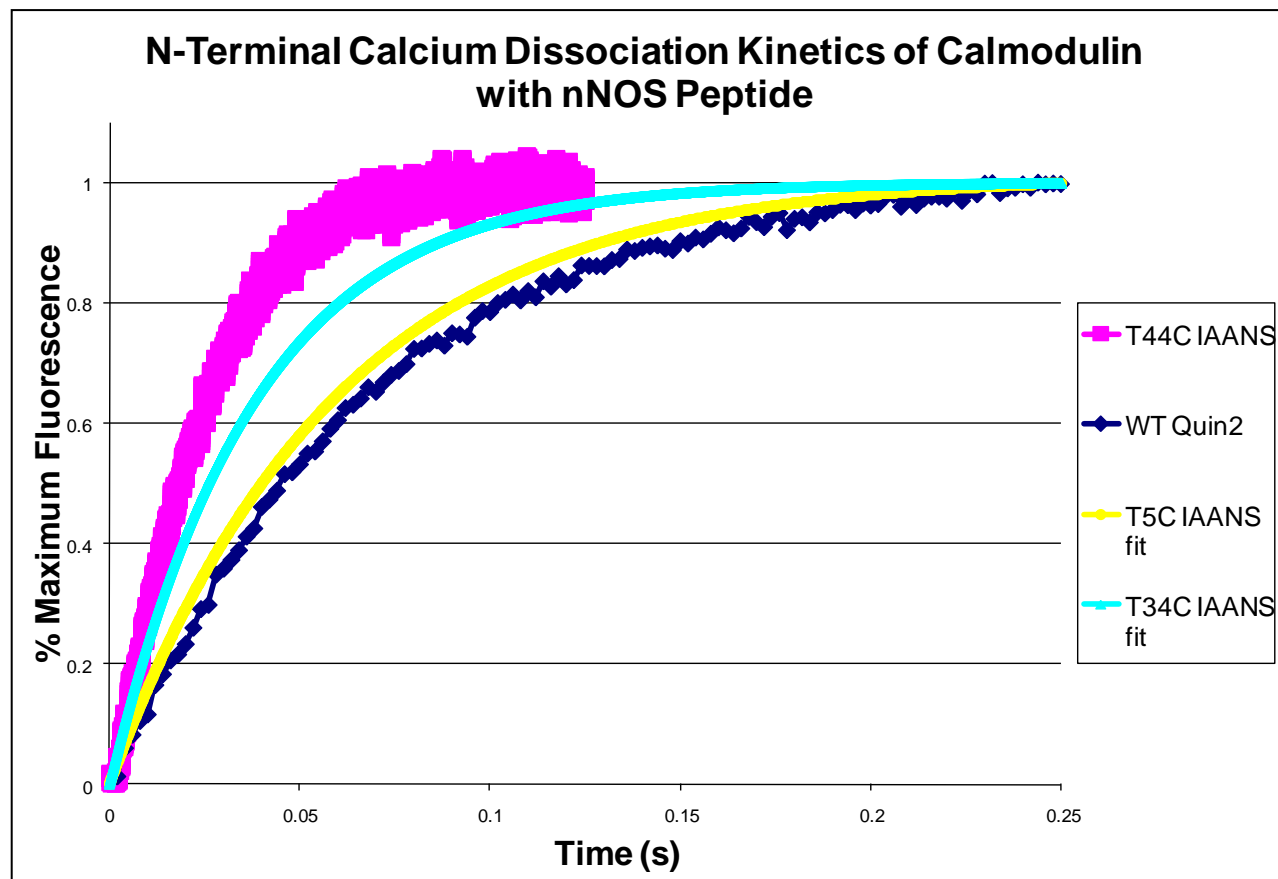


Figure 10

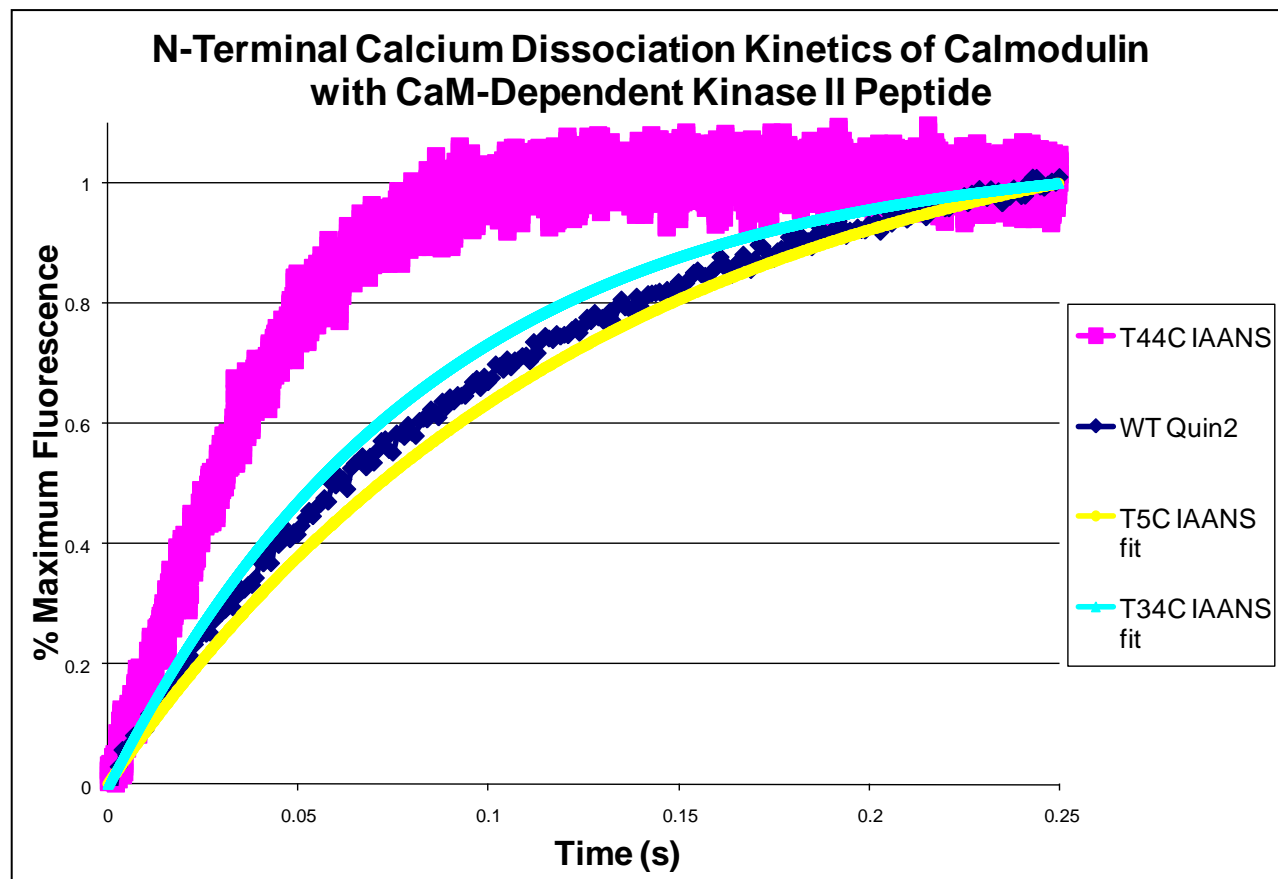


Figure 11

