Site-Directed Mutagenesis and Site-Specific Binding Analysis of Calmodulin (CaM)

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In partial fulfillment of the requirements for graduation with the Health Science Scholars Honors Degree in Biochemistry

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ABSTRACT

Calcium signaling is a major regulatory system in cells and a crucial part of cell biology. An important element in the decoding of intracellular calcium concentration into downstream processes is the ubiquitous and highly conserved calcium binding protein calmodulin (CaM) which can bind to and modulate the function of hundreds of different target proteins, regulating such processes as synaptic plasticity, gene expression and electrical signaling. The biophysical characterization of binding affinity and cooperative interactions between each of calmodulin's four EF-hand calcium binding sites is essential for understanding calcium signaling. Highly conserved amino acid sequence differences in the ion binding loops of the EF-hands give each site unique affinity for calcium. EF-hands are almost always found in pairs, where binding to one of the sites affects the affinity of the paired site. We have used spectroscopy to measure site-specific binding in each of the paired binding sites in the CaM N-lobe, along with site-directed mutagenesis, to study the contributions of individual amino acids to the ion binding affinity in the mutated site (cis effects) and in the neighboring site (trans effects). Of the twelve amino acids in the binding loops, five are different between Site 1 and Site 2. We constructed proteins with substituted individual residues from Site 1 to Site 2. CaM with the full Site 1 sequence in both Site 1 and Site 2 shows significant changes in affinity and binding characteristics in both sites. To investigate the contributions of the individual amino acid differences, we made intermediate mutants containing individual amino acid changes in Site 2. The cis-effects of the intermediate mutations on the mutated site, Site 2, seem to be independent and additive, whereas the trans-effects on the non-mutated Site 1 showed unexpected dependence on combinations of amino acid changes in Site 2.

1. IMPORTANCE OF CALMODULIN

1.1 What is Calmodulin?

It is late at night, but you are awake studying for tomorrow's neuroscience exam in university. You are quite tired, but continue to quiz yourself on the structures of the brain and ask yourself, 'What was the function of that region of the brain, again?' For a moment, you don't rack your brain for the answer to your quiz question. Rather, you pause, and think about the act of learning and remembering. You *are* in a neuroscience class, after all. Your mind slowly lingers away from tomorrow's quiz terms, and you begin to think about the concept of learning and memory. Isn't it amazing that the brain can recall information that was previously learned? There was once a time when you did not know that piece of information. It did exist; however, you were simply not aware of it. And after repeating the information to yourself a few times, you came to know and understand the information better and better. How is it that the brain is able to remember information that it was once told?

It is known that the protein calmodulin is involved in this process of remembering, as calmodulin is well known to be linked to learning and memory (Giese and Mizuno, 2013). But what exactly is "calmodulin?" Calmodulin is a protein that is abundant in all eukaryotic cells, and mediates many important processes in the body. Some of these processes include synaptic plasticity (related to learning and memory), smooth muscle contraction, metabolism, inflammation, ion channel regulation, and apoptosis. Evidently, calmodulin has *many* functions, some of which will be explored in this paper. But of particular interest to myself and the Aldrich laboratory, is calmodulin's ability to act as a "calcium sensor," because it is a

calcium binding protein. Calcium binding proteins act by binding to calcium, which "activates" them, allowing them to exert their downstream effects such as binding onto other proteins. What this means, is that calmodulin acts in a signal transduction system, where the influx of the second messenger calcium ions into a cell cause the binding of calcium to calmodulin, which causes this complex to be active and bind to downstream effector proteins. However, it has been found that calmodulin's affinity for calcium changes upon the alteration of amino acids in the calcium binding sites of calmodulin. These studies were implemented in the Aldrich lab. We employed a methodical mutation study method, known as "site specific and site directed mutagenesis" to create the exact, desired amino acid changes in the wild type calmodulin protein. The data from these studies was gathered, and will be discussed later in the paper. The importance of this research is to understand the molecular design of calmodulin, to hopefully assist with the creation of medicines that can bind to calmodulin and modulate its effects in relation to learning and memory.

1.2 Synaptic Plasticity

Calmodulin is a protein, which means it is a long chain of amino acids folded into a three-dimensional structure that is able to bind to other molecules and proteins, and exert changes in the cell. This protein is so unique, because it is able to bind to hundreds of other proteins, which means that it can be involved in many processes in the cell (Hoeflich and Ikura, 2002). However, calmodulin is famously known for its involvement in learning and memory.

In neuroscience, learning can be thought of as strengthening synapses due to patterns in activity that an individual executes (Martin et al., 2000). This is called long-term potentiation, or LTP (Bliss and Collingridge, 2003). When activities are repeated, the strength of the signal between two neurons in the brain increases as a long-term consequence. However, if the pattern of activity is halted, then there is no more communication between the two neurons, which subsequently decreases the signal transmission between the two neurons. This is called long-term depression, and is the opposite of what occurs during long-term potentiation. What has just been described reflects the brain's magnificent power to rewire itself, strengthening connections in the brain between neurons that are being used and weakening connections that are not being used. The brain allows the level of the chemical signal that is released between the synapses of neurons to change, which is known as synaptic plasticity (Bliss and Collingridge, 1993). This is the molecular basis of learning.

1.3 CaM Kinase Cascade

Now, how does calmodulin come in to play? For example, we can examine the CaM kinase cascade, which is involved in memory consolidation (Giese and Mizuno, 2013). As was stated earlier, calmodulin derives its importance from its ability to bind to other proteins in the cell and exert changes. In the CaM kinase cascade, the postsynaptic neuron receives the signal from the presynaptic neuron. This leads to the activation of NMDA (N-methyl-D-aspartate) receptors on the surface of the postsynaptic neuron. The NMDA receptor is a Ca^{2+} ion channel that binds the glutamate that was released by the presynaptic neuron. Thus, when glutamate binds to the NMDA receptor, the channel opens and allows an influx of cations into

the cell, which then dislodges ions such as Mg^{2+} and Zn^{2+} that are blocking the pore in the NMDA ion channel. Once these ions are removed from the pore, Ca^{2+} ions flow into the cell. Once the calcium ions are in the cell, they bind to calmodulin. This activates calmodulin, which then allows the calcium-calmodulin complex to bind to kinases, such as CaMKK, which then binds to other kinases CaMKI, CaMKIV, etc. Then, CaMKIV phosphorylates other proteins, such as CREB and CBP in the nucleus of the cell, which causes gene transcription to occur. Then, these genes translated into proteins that specifically target activated synapses, and lead to memory solidification (Giese and Mizuno, 2013).

1.4 AMPA Receptors

Another example of a calmodulin pathway related to synaptic plasticity involves the insertion of new AMPA receptors in the membrane of the postsynaptic neuron (Lu et al., 2001). This pathway involves the concept of long-term potentiation. When glycine binds to postsynaptic NMDA receptors, an influx of Ca^{2+} results. This calcium influx leads to the insertion of AMPA receptors in the membrane, which subsequently allows for the cell the experience an even stronger signal from the presynaptic neuron in the long-term (Lu et al., 2001). This vivid demonstration of synaptic plasticity in action is a molecular description of how learning takes place.

Furthermore, calmodulin is involved in the insertion of AMPA receptors into the postsynaptic membrane through Ca^{2+}/calmodulin-dependent protein kinase II, or CaM kinase II (Stefan et al., 2008). In the molecular cascade, CaM kinase II phosphorylates AMPA channels at a particular amino acid (serine 831). This increases the conductance of the AMPA

channels that bind glutamate, which means that the channels are more sensitive to a signal. Subsequently, long-term potentiation and increased synaptic strength are the results (Lisman et al., 2002). Furthermore, links have been found between improper CaM kinase II regulation and Alzheimer's disease, which makes the study of calmodulin and CaM kinase II is medically relevant (Yamaguchi, 2005).

2. MEDICAL RELEVANCE OF CALMODULIN

2.1 Alzheimer's Disease

Alzheimer's disease, a gradual mental deterioration that typically occurs in older individuals, results in patient forgetfulness and memory loss (Khachaturian, 1985). This disease is scientifically associated with amyloid-β plaques and neurofibrillary tangles. Amyloid-β plaques are aggregations of protein fragments in neurons, which are toxic to brain cells. Neurofibrillary tangles are fibers of hyperphosphorylated tau protein, which are involved with the microtubules in nerve cells. Microtubules are used to shuttle materials around the cell from one place to another.

In Alzheimer's disease, the accumulation of amyloid-β plaques between neurons, as well as the accumulation of neurofibrillary tangles within neurons, overwhelms the body and results in the typical Alzheimer's disease symptoms, such as confusion and memory loss. It has been experimentally confirmed that certain types of calmodulin binding proteins, such as amyloid-β protein precursor, β-secretase, presenilin-1, ADAM10, etc., are involved in the formation of amyloid-β plaques (O'Day et al., 2015). Therefore, the more we understand

about the calmodulin protein, the more we can learn about its relevance to Alzheimer's disease. This will put scientists in a better position to devise a medication that can act with calmodulin, or its binding proteins, in an effort to try and combat the symptoms of Alzheimer's disease.

2.2 Long-QT Syndrome

Another medical disease involved with the protein calmodulin is long-QT syndrome. In this disease, the patient's heart beats arhythmically, quickly, and chaotically (Schwarts, 2005). Because of this irregular heartbeat, the patient may suddenly die. Long-QT syndrome is named as such because when a heartbeat wave is examined on an electrocardiogram, there is a section of the curve called the QT interval. In long QT syndrome, the QT interval in the electrocardiogram is elongated, and thus this disease is named long-QT syndrome (Schwartz, 2005). The symptoms of long-QT syndrome include fainting (especially during stressful or situations), seizures, or sudden death. Long-QT is hereditary, and is more likely to occur in individuals that have a family history of the disease (Schwartz, 2005). Additionally, the risk factors for the disease include liver or renal dysfunction, cardiovascular disease, or electrolyte imbalances. In consideration of the pathophysiology of long-QT syndrome, it is found that all types of long-QT syndrome are linked to an abnormal repolarization of the heart. As a result, the refractory period of the heart muscle cells is different, and leads to early after-depolarizations in the cells, which leads to ventricular arrhythmias. The molecular mechanism for this behavior has been identified, because certain types of long-QT are associated with mutations in calmodulin. When there are mutations in calmodulin, then the inactivation of L-

type calcium channels and currents may occur. Specifically, these L-type calcium channels are held open, or reopened, during the plateau phase of the cardiac action potential. Subsequently, this leads to the sustained flow of calcium into the cardiac cell, which leads to arrhythmic behavior in ventricular myocytes, or heart cells (Limpitikul et al., 2014). And as stated before, it is more likely for long-QT syndrome to be exhibited, or result in the sudden death of a person, when the individual is exercising or emotionally excited. Since adrenaline activates the L-type calcium channels, which are already impaired with respect to repolarization, this puts a huge strain on the heart muscle, and leads to sudden patient death.

Mutations in calmodulin, such as the recently discovered D132H mutation, can lead to long-QT syndrome by affecting the calcium current in human cardiac cells. There are many case studies of young infants or toddler patients who went into cardiogenic shock or cardiac arrest, only later to resuscitated and discover, after genetic testing, that they had long-QT syndrome (Pipilas et al., 2016). In this 'calmodulinopathy,' the calmodulin mutations result in a calmodulin where calcium can only weakly bind to the C domain of the protein. Additionally, calmodulin mutations result in some structural malformations of the protein itself. This makes sense, because protein tertiary structure is dependent upon its secondary and primary structures, which are dependent upon the amino acids sequence. When calmodulin can only weakly bind to calcium, then the calcium/calmodulin complex is not as effective in activating other target enzymes. The voltage gated calcium channels are impaired, and Ca^{2+} dependent inactivation of voltage-gated Ca^{2+} current is impaired as well, leading to long-QT syndrome (Pipilas et al., 2016).

2.3 Calmodulin as a Drug Target

The above discussions of calmodulin's associations with medical diseases such as Alzheimer's and Long QT syndrome, are what make calmodulin an interesting therapeutic target. For example, research has been done in an effort to create a drug that targets CaM kinase II (Banyasz et al., 2011). The traditional method for dealing with cardiac arrhythmias is to implant a cardioverter defibrillator. Currently, this is considered as the most effective way to deal with cardiac arrhythmias. However, it would be very clinically useful to have medications to treat cardiac arrhythmias, because there are certain cardiac arrhythmias that cannot be treated with implantable cardioverter defibrillators. There are some medications that target cardiac ion channels, however there has only been moderate success with these treatments, some of which have been found to actually increase mortality. Thus, researchers have begun to look at molecular drug targets that act upstream of ion channels, such as CaM kinase II (Banyasz et al., 2011). If CaM kinase II is inhibited, then this can be a treatment for several types of heart diseases. This is because high CaM kinase II levels have been found in individuals who have failing hearts (Banyasz et al., 2011). Additionally, CaM kinase II plays a key role in the regulation of the heart. If medications can be constructed to work with CaM kinase II, a protein the binds calmodulin, then perhaps they can be used as a treatment for certain heart conditions.

The protein calmodulin shows tremendous promise for being a drug target. Calmodulin is involved in many processes in the body, and is found in many locations within the body, such as in the brain and heart. Calmodulin has over 200 binding partners, which reflects the sheer magnitude of importance for the molecule (Hoeflich and Ikura, 2002). Many

target proteins and enzyme are only able to function once they are bound to calmodulin. Therefore, it is very worthwhile to better understand this unique protein. If additional information about calmodulin can be uncovered, then scientists can not only better understand the molecule, but also be in a better position to devise drugs and medicines that act on calmodulin to modulate its effects.

3. INTRODUCTION

3.1 Rational and Purpose

Previously, the medical and scientific importance of calmodulin was examined. This discussion set the stage for the importance of the research that the other members of the Aldrich lab and I conducted. The overall goal was to better understand how individual amino acids contribute to the calcium affinity of the calmodulin protein. By altering certain amino acids of calmodulin, the binding affinity of the calcium binding site would change. This information allowed us researchers to understand the importance of certain amino acids in the protein.

The purpose of this research is to examine how amino acid sequences, and adjacent sequences as well, can affect binding. The primary technique used in this research, known as site directed mutagenesis, involves altering the gene for calmodulin at specific sites. This technique is very powerful, as it allows for the creation of whatever protein mutant is desired. By creating chimeras and mutated versions of the calmodulin protein, the Ca^{2+} binding affinity of the protein is altered in very unique and specific ways. In taking a detailed

approach with site specific mutagenesis, we can be thorough in our understanding of the CaM protein.

3.2 Calmodulin Structure

The calmodulin protein has a very symmetrical structure. It has four calcium binding sites, two of which are localized towards the N lobe, and two of which are localized towards the C lobe. Between the N lobe and C lobe of the protein (which are two very distinct halves), lies a middle linker region. Each of calmodulin's four binding sites, or EF hands, is able to bind a Ca^{2+} ion (Figure 1). All of these four EF hands are actually very similar in sequence to one another. Thus, the specific amino acid differences between the four EF hand binding loops are of particular interest in our research. Amino acids are known to have very unique properties, and thus when altered, could affect the entire protein function as a whole. In our research, we are only considering the N lobe half of calmodulin for now. This is possible to do because the N lobe and the C lobe form separate, distinct globular shapes when the protein is in its conformational shape in the tertiary structure. Thus, all of the mutants that will be listed in this research paper refer to mutations that were made in the N lobe of calmodulin.

3.3 Background Information

The background behind this project has mainly to do with the fact that calmodulin binding assays have previously been done and explored. However, most of these assays involve examining the total binding of calmodulin to Ca^{2+} (Linse et al., 1990). This means

that the measurements obtained involve the entire calmodulin protein, and act as only an effective summation of the binding affinities for all the four individual binding sites[2]. Although the curves that are generated with these methods appear to be scientifically sound, they are not very representative of the calmodulin protein. This is because each of the four calmodulin binding sites has its own unique affinity for Ca^{2+}, based on the amino acid sequence context. Each of the four binding sites has its own unique apparent affinity, and this cannot be represented on a curve that depicts calmodulin total binding. The previous total binding curves are insufficient, because the association constants and cooperativity factors vary by several orders of magnitude. Thus, the need for site specific binding assays of the protein calmodulin emerged, and our lab started to conduct site-specific measurements of terbium and calcium binding to calmodulin and calmodulin fragments (Greeson-Bernier et al., 2013).

4 MATERIALS AND METHODS

4.1 CaM Expression and Purification

To conduct the site directed mutagenesis itself, polymerase chain reaction (PCR) was used. Then, to generate the desired mutated calmodulin protein, a bacterial expression system was used. The gene sequence for N lobe of the protein CaM was cloned into an expression vector. The CaM protein itself was then expressed in bacterial BL21 cells, which were grown in LB media. After an initial ammonium sulfate purification, high performance liquid chromatography, or HPLC, was used to purify the protein. Calmodulin was isolated by using

protein purification techniques. The primary techniques utilized include ammonium sulfate washes, and purification using high performance liquid chromatography (HPLC). The types of columns used during the protein purification process were dependent upon how well the previous column was able to separate the protein. Some columns that were used include Desalt, Anion Exchange, C18, and Superdex. Once the desired protein was isolated, a gel was run to confirm the purity of the protein sample, and to assure that there were no contaminants. After a Final Desalt column, the CaM was stored in 5 mM HEPES Additionally, gels are usually run during the protein purification process (between running HPLC columns), to check for protein purity through the procedure. The concentration of protein was determined using absorbance spectroscopy, specifically looking at 280 nm absorbance.

4.2 Solutions

Teflon vials were used to store the lanthanide (terbium ion, Tb^{3+}) solutions. Dilutions of the lanthanide solutions were made, ranging from 1μM to 10 mM. Additionally, dilutions of HCl solutions were also prepared for the experiment, and stored in Teflon vials as well. The base solution for the experiments is an MES solution, which contains 2-(N-morpholino)ethanesulfonic acid, 6 mM KCl, 136 mM KOH, and 5 mM HEPES.

4.3 Site-Specific Binding Measurements

A fluorometer (Photon Technologies International) was used to perform site-specific binding measurements. The light excitation source was a Xenon flash lamp, which pulsed

light at a frequency of 100 Hz. Light of 292 nm was used to excite the tryptophan that was engineered into CaM, which would then transfer energy to the bound Tb^{3+} ions. The energy transfer is localized to the site nearby the tryptophan. An average of 10,000 light pulses were emitted per trial.

The protein binding affinity was measured by conducting site specific binding experiments with terbium ions, which are known to act as excellent calcium substitutes for calmodulin (Martin and Richardson, 1979). The ability to use terbium ions as a replacement for calcium ions is extremely useful, because terbium ions are luminescent, allowing their fluorescent decay to be registered by proper instruments.

This project is unique in that terbium ions (Tb^{3+} ions) can serve as substitutes for Ca^{2+} ions. This has been discovered in prior research, and is widely known and accepted (Martin and Richardson, 1979). Terbium ions have luminescent properties, which allows for signals to be measured. This is extremely convenient for this project, because Ca^{2+} ions, which normally bind to calmodulin, do not have luminescent properties. Thus, since calmodulin can bind Tb^{3+} as a substitute for Ca^{2+}, the luminescent properties of Tb^{3+} can be utilized in visualizing signals.

While the mutated CaM protein was being generated, a noninvasive tryptophan residue was engineered into the amino acid sequence for only one binding site in the calmodulin protein. In the spectrofluorometer, light with a wavelength of 292 nanometers is emitted, which excites this tryptophan residue. Then, this tryptophan residue transfers its energy to a nearby Tb^{3+} ion, which is in that particular binding site of calmodulin where the tryptophan residue was engineered. Then, the Tb^{3+} emits light with a wavelength of 545 nanometers, which is then detected by the spectrofluorometer. The mathematical analysis

program that is used in our lab to analyze the data is able to separate the two different signals that the spectrofluorometer received: one signal from the Tb^{3+} that is bound to the protein, and another signal from the Tb^{3+} that is free in solution. In summary, the program can separate the "bound" signal, from the "unbound" signal.

4.4 Exponential Decay Fitting

During each Terbium titration experiment (each "trial"), a collection of exponential decays are generated. These decays are then fit using the Markov Chain Monte Carlo (MCMC) method. The data that is collected during the experiment has two components: the signal from the Tb^{3+} that is bound to the CaM, and the signal from free Tb^{3+} in solution (or, "un-bound"). The former is known as the "slow component" meaning that the signal decays slowly, while the latter is known as the "fast component," meaning that the signal decays quickly. Because there are these two components, a double fitted exponential results. The MCMC is able to effectively separate these two differing signals, allowing for the generation of sigmoidal dose-response curves. The dose response curves were generated using the amplitudes of the terbium luminescence decay at increasing terbium concentrations.

The raw data that is generated with the PTI machine is a graph that depicts concentration dependent site specific Tb^{3+} luminescent decays. These graphs are luminescent decays over time. Then, this data is analyzed using an algorithm (Markov Chain Monte Carlo) and normalized amplitude curves are generated. These curves are sigmoidal, which exhibit the cooperative binding of calmodulin (Valeyev et al., 2008). Cooperative binding is when the protein binds to a substrate, which increases the protein's affinity to bind more substrate in the

other active sites. The axes on these curves have Normalized Amplitude (on the Y axis) as a function of Added [Tb^{3+}] (on the X axis).

The differences between these curves for each of the mutants was then analyzed and compared. By methodically mutating specific amino acids in the EF hand structures of the N lobe, we can determine the contribution of each amino acid to Ca^{2+} binding.

4.5 Definition of Wild Type

Most of our lab's research is concerned with creating calmodulin mutants, also known as chimeras. Our lab systematically chooses which chimeras to make. We define the "wild type" calmodulin as having no amino acid changes from the wild type calmodulin sequence, except, with a noninvasive tryptophan residue inserted into the amino acid sequence at position 7 of the EF hand binding loop. This was done so that measurements could be made with the terbium ions. Without the tryptophan residue, a signal from the bound terbium cannot be recorded. This is because the bound terbium would emit the same wavelength of light as the free terbium in solution, because the tryptophan would not be there to excite the bound terbium. This experiment relies on the fact that the decay time constant shifts upon terbium binding (Figure 2).

4.6 Chimera Nomenclature

Because so many similar, but slightly different mutants are created for this project, it is very important to convey relevant information when naming the chimera mutants. For the

nomenclature of the chimeras, the first EF hand of calmodulin is called Site 1, the second EF hand of calmodulin is called Site 2. Also, our lab calls the actual amino acid sequence in the first EF hand in calmodulin Sequence A, and the sequence in the second EF hand in calmodulin Sequence B. As stated previously, only the N lobe half of calmodulin is being considered in our current research, and exploration of the C lobe half of calmodulin is a future direction. Thus, using this nomenclature, the wild type for our lab had Sequence A in Site 1 and Sequence B in Site 2 (Figure 3).

5. RESULTS AND DISCUSSION

5.1 Primary Experiments

Our lab has generated many mutants, but only a select few will be discussed in this thesis. For example, our lab has generated a chimera mutant when Sequence A is in both Site 1 and Site 2. What this means, is that this chimera mutant has the amino acid sequence from the first EF hand into both EF hand sites. When using a reporter (tryptophan residue in Site 1), this causes a right shift in the terbium binding curve (Figure 4). Our lab's goal is to now look at the amino acid sequence in detail, try to ascertain the reasons behind this shift, and to try to determine which amino acids are responsible for this shift. The shift of the graph indicates that this particular mutant of calmodulin, where Sequence A is in both Site 1 and Site 2 of the protein, has a higher affinity for calmodulin.

Now, in an effort to try and pinpoint exactly which amino acid, or cluster of amino acids, could be responsible for this shift, our lab generated more chimera mutants. Firstly, it is

important to note that between the amino acid sequence in the first EF hand (Sequence A) and the second EF hand (Sequence B), there are only 5 amino acids that are different. Additionally, 3 of these different amino acids are clustered right beside one another in the sequence, so we decided to create mutants with this sequence of three amino acids first. To do this, we created a chimera that contains Sequence A in Site 1, and Sequence B in Site 2, except in Site 2, instead of having the full, wild type Sequence B, we have changed the DFP amino acids to TTK. This is because those (TTK) amino acids are the amino acids that are at the same position in the EF hand binding loop in Site 1 (Figure 5). In summary, what we have done essentially is only taken a portion of Sequence A, and inserted it into Sequence B. Once we did binding assays on this mutant protein, we were able to see a shift that nearly completely overlapped with the previously discussed mutant (where Sequence A is in both Site 1 and Site 2 of the protein). What this suggests, is that one of the three amino acids, or a combination of those, is responsible for the shift that was generated in the graph seen earlier.

In conclusion, the apparent affinities of the N lobe can be changed by mutating the adjacent site of the protein, which was seen in the Sequence A chimera. Additionally, the DFP to TTK mutation in Sequence B overlaps with the Sequence A chimera, suggesting that the affinity is similar.

Next, we examined the difference due to all 5 residues individually, and determined the contribution of each of the five different residues in Site 1 and Site 2. From the group of three amino acids, we examined each of the three individually, and made mutants where only one of those three is altered at a time.

5.1 Secondary Experiments

Two sets of experiments were run in an effort to investigate the individual amino acid contributions across paired EF-hands in calmodulin. For the nomenclature, the wild type Site 1 contains amino acid sequence "a," while the wild type Site 2 contains amino acid sequence "b." So for example, chimera "aa" is a calmodulin protein that contains the wild type amino acid sequence of Site 1 in both Site 1 and Site 2. Moreover, the location of the asterisk "*" conveys information about the site that the reporting tryptophan is located in. For example, if the asterisk is placed "a*b," then for this wild type calmodulin chimera, the tryptophan reporter is located in Site 1. However, if the asterisk is placed "ab*," then for this wild type calmodulin chimera, the tryptophan reporter is located in Site 2.

To understand the aa chimera results, special constructs were created. Figure 6 depicts the steps that were used to create the chimera mutants. The pink slice in the pictures shows that the amino acid at that location matches its counterpart in sequence a. Experiment 1 examines the three groups of mutations that together make the aa chimera. Experiment 2 specifically examines the abTTK mutant, by breaking up this group mutation into three smaller mutations: abD-T, abF-T, and abP-K.

The goal of Experiments 1 and 2 are to understand the trans and cis effects of ion binding. Using site-directed mutagenesis, the contributions of individual amino acids to the ion binding affinity in the mutated site (cis effects) and in the neighboring site (trans effects) can be studied.

In Experiment 1, the cis and trans effects of the aa chimera were deconstructed by looking at intermediate constructs (Figure 7). Regarding the trans effects, it was found that

positions 9 and 10 play a large role in trans effects, as can be seen because the a*bTTK binding curve just about overlaps with the a*a binding curve. Additionally, the N60D mutation causes a steeper slope in the graph related to Site 1, which is a sign that this amino acid may be related to cooperativity between the sites (Figure 7b). Regarding the cis effects, each mutation seemed to be additive, and the summation of the mutations shifted the curves enough to sufficiently overlap with the aa* binding curve (Figure 7e). Also, adding a positive charge, as in mutation A57K to the binding loop greatly increased the binding. This interesting discovery comes unexpectedly, because the binding loop is binding onto a positive cation, and it would seem unlikely that adding a positive charge to the binding site would increase the binding. Furthermore, adding a negative charge, as in mutation N60D, to a directly coordinating position yields little change in binding. Evidently, charges in proteins may not behave straightforwardly. Unexpected results may arise, and with the accumulation of future research, the reasons behind these unique findings may be identified.

Experiment 2 studied the cis and trans effects of the TTK mutation. Regarding the trans effects, no individual amino acid was able to account for the shifts in the binding curves of a*bTTK or a*a. Notably, the opposite phenomena of Site 1 was seen in Site 2, where the mutations have a combined action on the neighboring site that is greater than the sum of the individual mutations. This synergistic result can be seen in examining the graphs of Figure 8A, 8B, and 8C, because each of the individual mutations does not shift the binding curve by very much. However, the whole mutation is able to shift the binding curve by quite a bit, more that the sums of all of the individual mutant binding curve shifts. Regarding the cis effects, the single point mutation D-T blocks the binding. This can be seen on the binding curve in Figure 8D, because the trace of the curve is misshapen. Additionally, the F-T and P-

K mutations have relatively small effects, especially when considering the properties of the amino acids. The switch from P to K involves a switch from a nonpolar amino acid to one that is polar and positively charged. This unique finding is similar to the one that was discussed above regarding Experiment 1. Amino acids may behave in ways differently than expected.

6. CONCLUSIONS

6.1 Conclusions

In summary, each amino acid in the binding loop has additive binding contributions to its own binding site that it is located in, as well as non-additive binding contributions to its neighboring site. As seen in Figure 9A, the intermediate mutations that were constructed each contribute a small amount to the overall shape of the aa* chimera binding curve. Thus, these amino acids have independent, yet additive roles in controlling cis effects. However, when all the mutations are together, as seen in Figure 9B, there is a large shift in the binding curve. This suggests that a simultaneous, combination of amino acid mutations is crucial in determining trans effects.

Each amino acid in the calmodulin binding loop plays a role in determining the local binding within the site that the amino acid is located within, as well as plays a part in indirectly determining the binding in the neighboring site. This notion further reinforces the idea that proteins are extremely complex biological units, that function in three-dimensional space in unpredictable ways. Through experiments and analysis we can attempt to collect information regarding how the protein behaves in certain situations and circumstances. We

can then take these findings and try to better understand the whole picture of how the protein functions alone, and when bound to other molecules and proteins.

6.2 Future Directions

In the future, our lab plans to investigate the cis effects of combined mutations, dissect the cis and trans effect of the bb chimera, study the entire C-lobe EF-hand chimeras, and eventually, construct full CaM chimeras with all four of the calcium binding sites. Conducting the above experiments will allow for our laboratory to have extreme insight regarding the importance of the amino acids and their positions in the calmodulin protein, unparalleled to any other laboratory in the world. We will be intimately familiar with the calmodulin protein, and will hopefully be able to answer some of the world's previously unanswered questions.

6.3 Final Statements

The goal in understanding how the calmodulin protein functions and operates is to be able to target calmodulin therapeutically. We hope that one day our findings regarding calmodulin binding affinities will be used by scientists and researchers who work in drug-screening and drug creation. Ideally, our findings will be of use to these other scientists around the world, who may be able to successfully devise a medication that can be used to heal some of the medical issues that were mentioned in the beginning of the paper, such as Alzheimer's or Long-QT Syndrome.

Science is a team-based effort, and hopefully our piece will be considered as a useful contribution.

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8. FIGURES AND TABLES

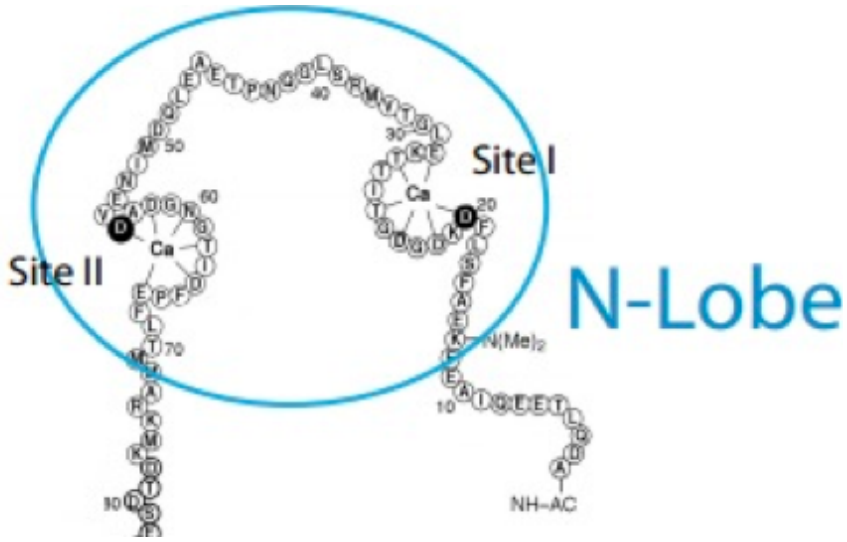

Figure 1. This is a depiction of the amino acid sequence for the N Lobe half of calmodulin. Displayed clearly are Site 1 and Site 2 of the protein.

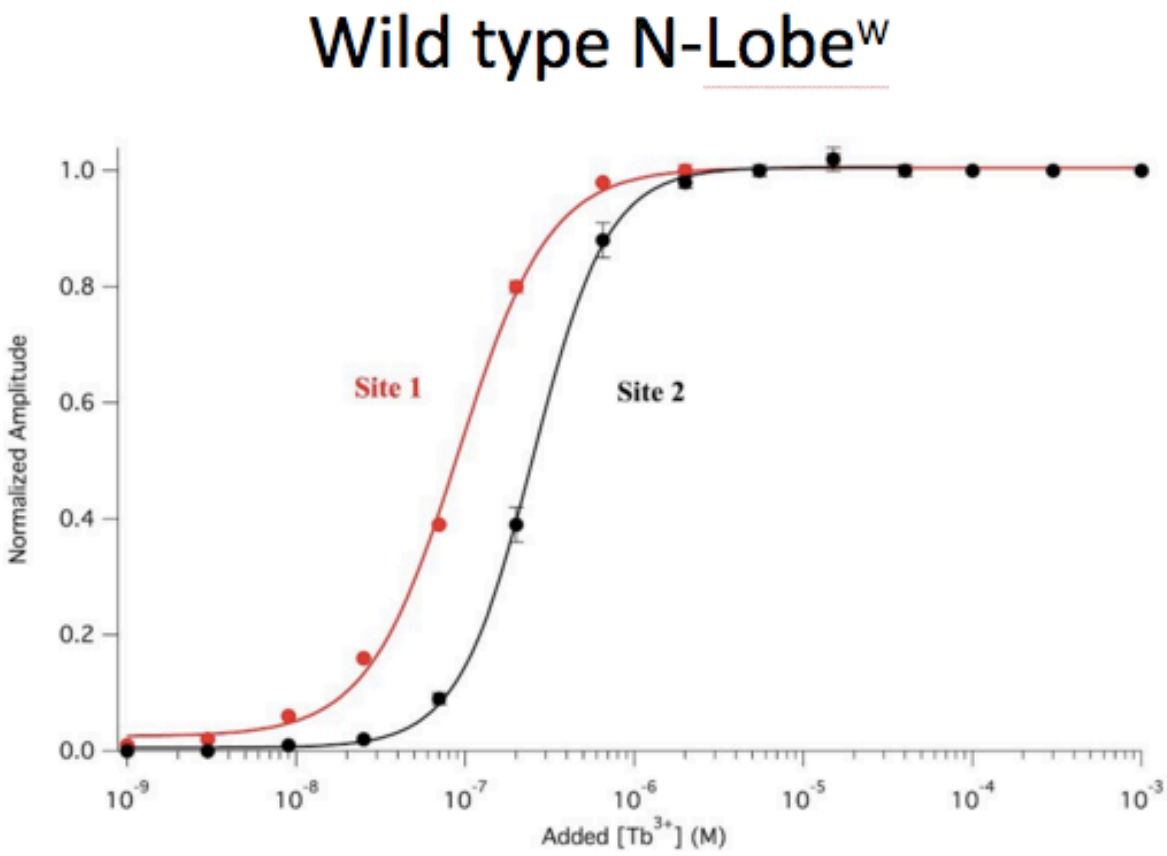

Figure 2. Normalized amplitude verses terbium concentration for the wild type N lobe calmodulin. In red, the reporter tryptophan has been engineered in Site 1, while in black, the reporter tryptophan has been engineered into Site 2. Some of this data was collected by Margaux Miller and Suzanna Bennett.

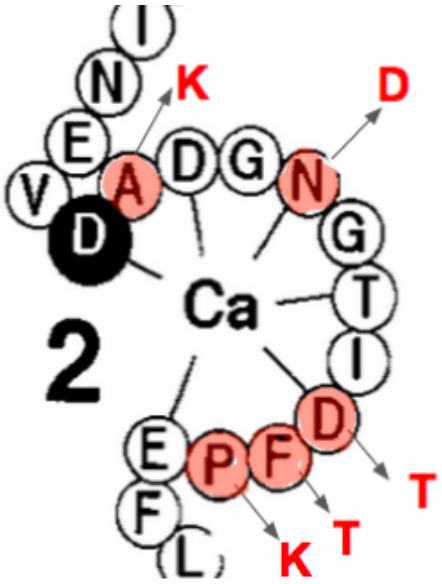

Figure 3. Only Site 2 is depicted here. Highlighted in red are the amino acids that differ between Sequence A and Sequence B. The arrows point to the corresponding amino acids in Sequence A that are in those same positions.

Sequence a chimera is right shifted from wild type N-Lobe[W]

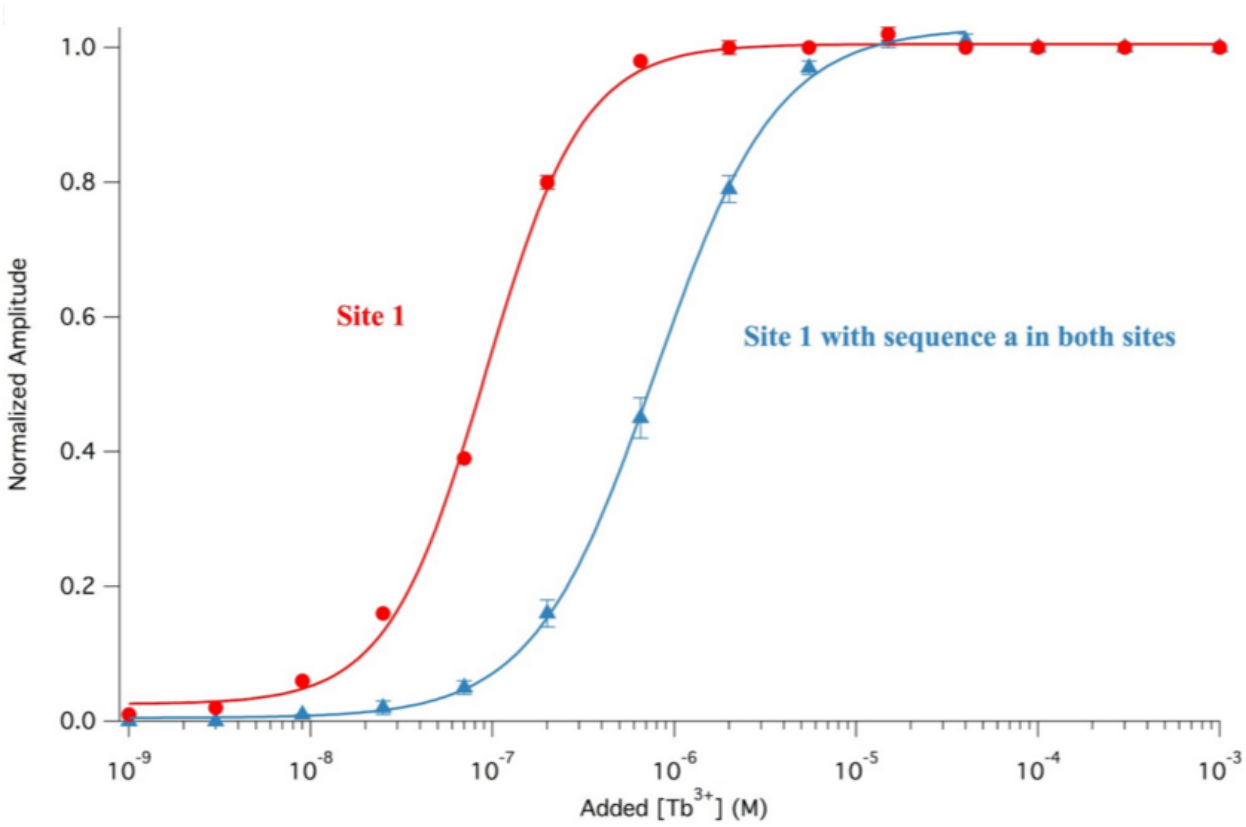

Figure 4. Normalized amplitude verses terbium concentration for the Sequence A chimera of calmodulin. In red, the wild type is depicted, with the reporter tryptophan engineered into Site 1. In blue, the mutant is shown, which contains Sequence A in both Site 1 and Site 2 of the protein. Some of this data was collected by Margaux Miller and Suzanna Bennett.

DFP to TTK mutation in sequence b overlaps the sequence a chimera

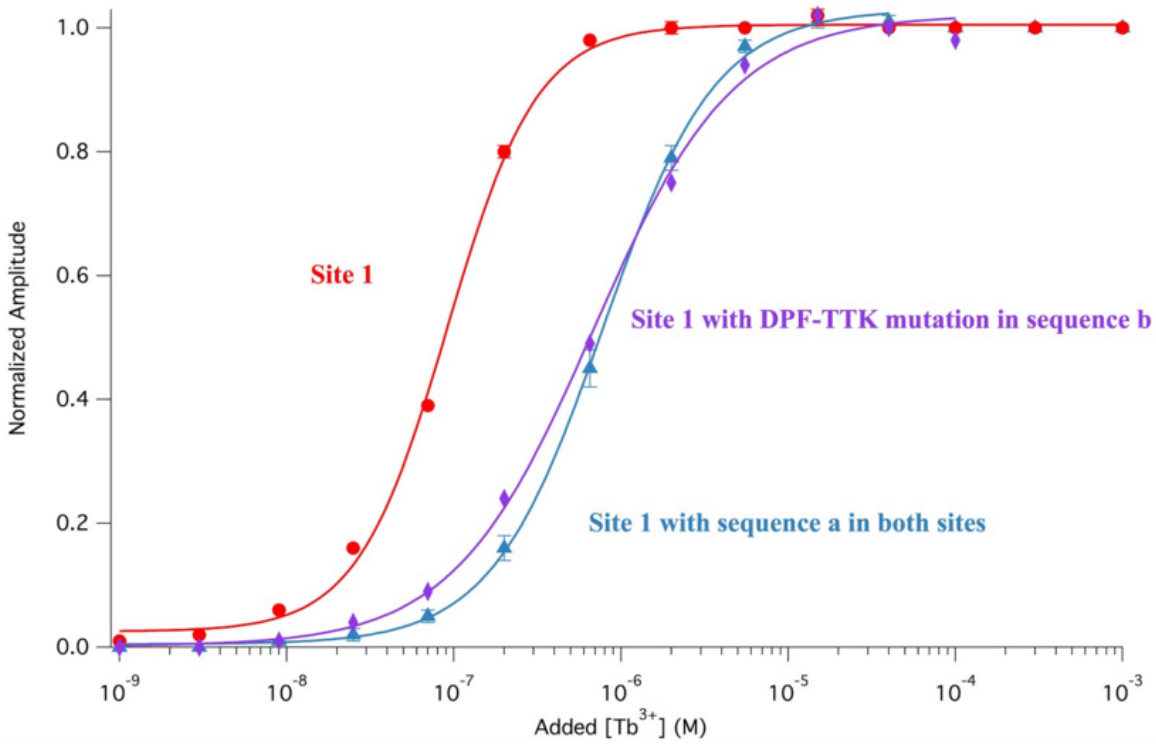

Figure 5. Normalized amplitude verses terbium concentration for the DFP to TTK chimera of calmodulin. In red, the wild type is depicted, with the reporter tryptophan engineered into Site 1. In blue, the previous mutant is shown, which contains Sequence A in both Site 1 and Site 2 of the protein. In purple, the new mutant is shown, which contains the DFP to TTK mutation. Some of this data was collected by Margaux Miller and Suzanna Bennett.

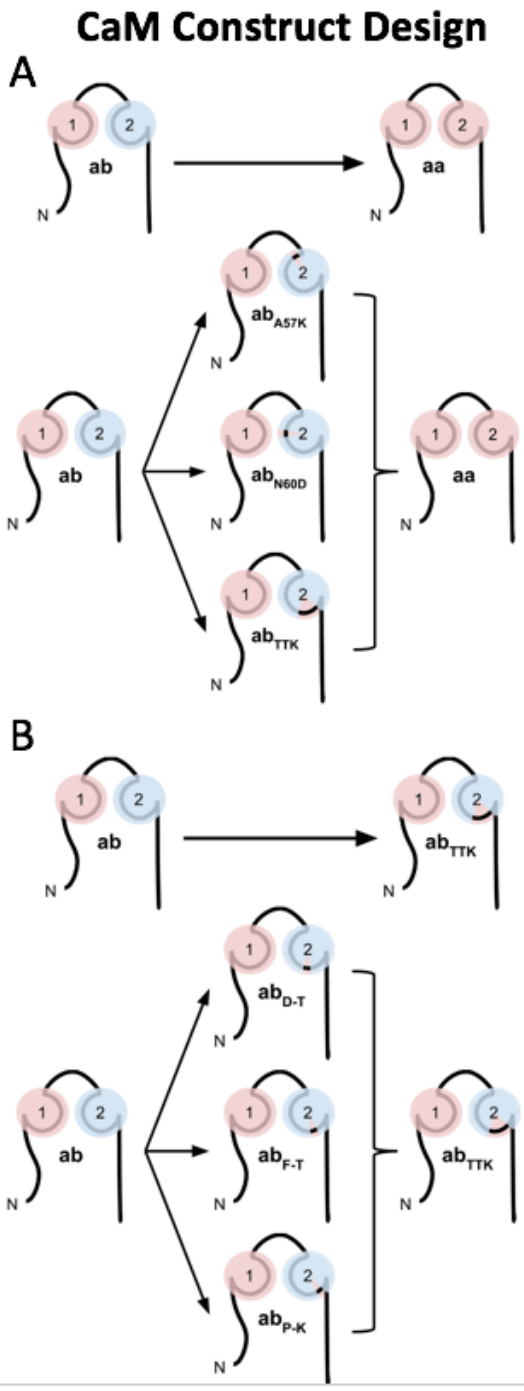

Figure 6. (A) Experiment 1 outline: Constructs made containing just one of the three groups of mutations that together made the aa chimera to dissect aa results. Middle column shows the three constructs used in this initial round of experiments with the mutation used noted as a subscript of the Site 2 sequence letter "b". In each construct, the loop position mutated is shown as a pink slice indicate the identity of that amino acid matches its counterpart in sequence a. **(B)** Experiment 2 outline: ab_{TTK} results prompted further investigation in both sites. Constructs made testing individual contributions of D-T, F-T, P-K mutations to each site. Data collected by Margaux Miller and Suzanna Bennett.

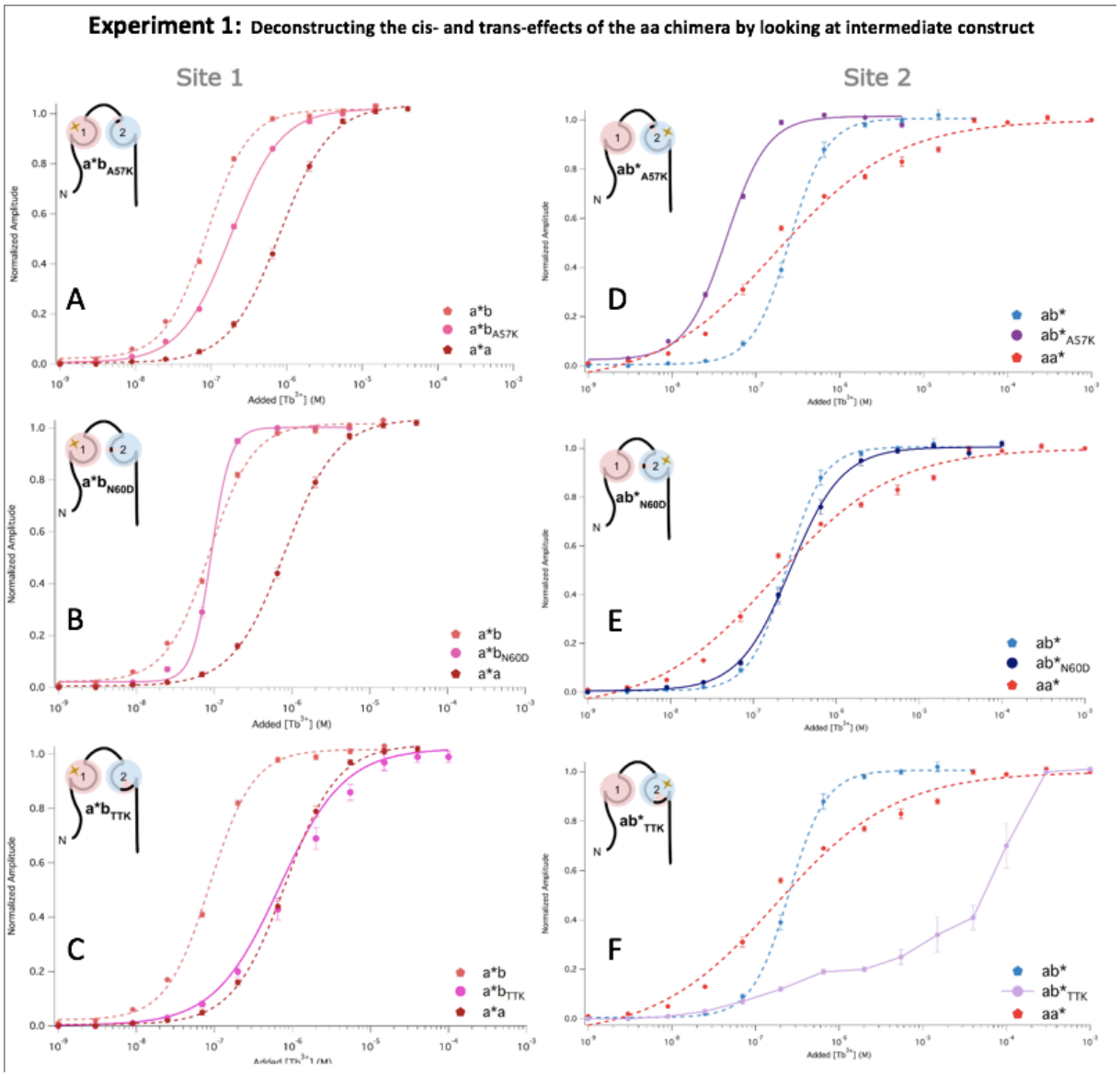

Figure 7. In each of these intermediate constructs, Tb^{3+}-binding was measured in Site 1 **(A-C)** and Site 2 **(D-F)**. Following the format in Fig. 2, the mutation is noted in the subscript. For reference, the binding curve for the native sequence and the aa chimera for each site shown as dashed lines, , a*b and a*a **(A-C)** and ab* and aa* **(D-F)**. Data collected by Margaux Miller and Suzanna Bennett.

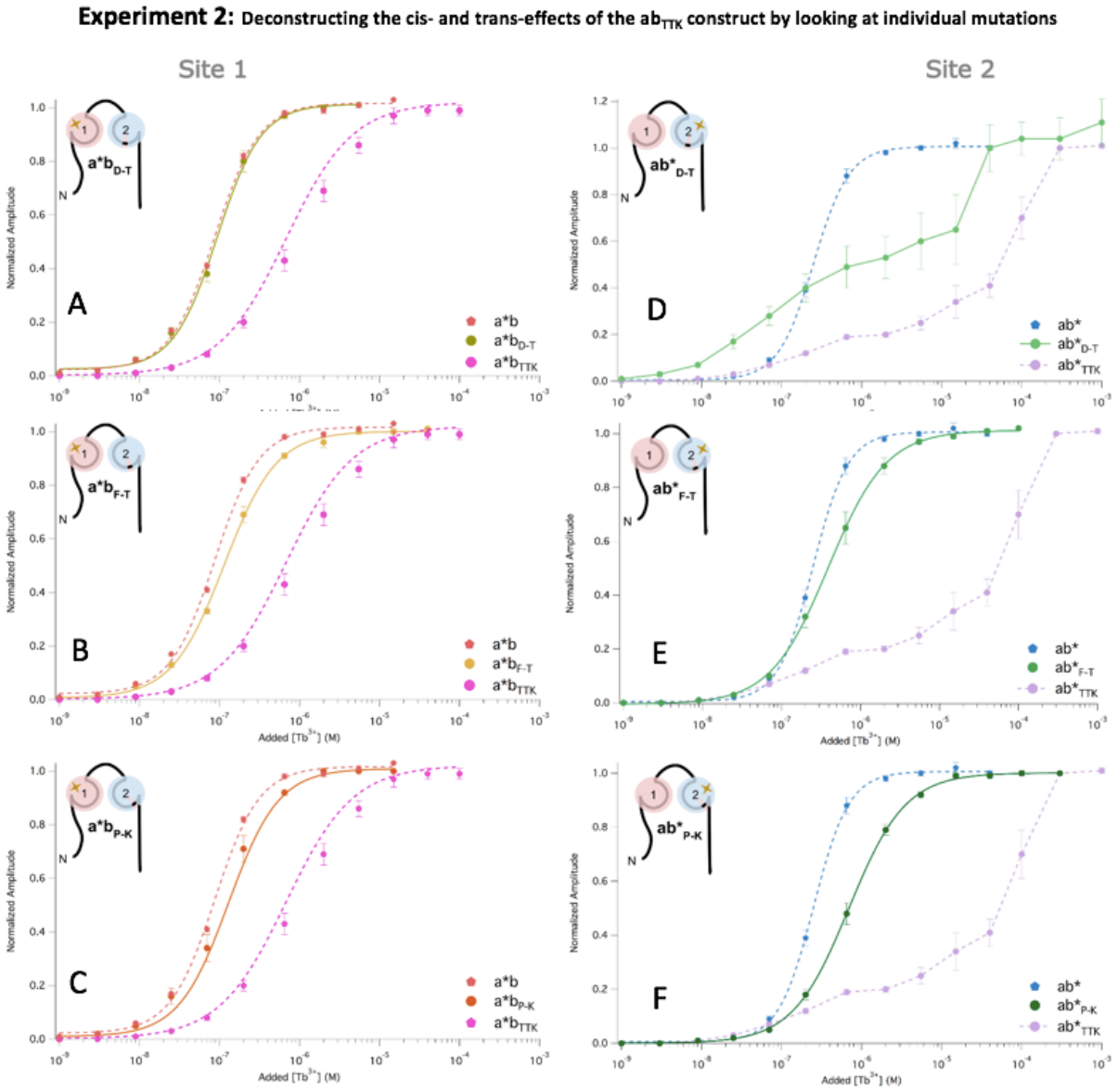

Figure 8. (A) Cis- and trans- effects of the TTK mutation **(Fig. 3C, 3F)** prompted further investigation, lead to the construction of mutants with individual amino acids changed. Following the format in Fig. 2, the mutation is noted in the subscript. For reference, the binding curve for the native sequence and the TTK mutant are shown as dashed lines, a*b and a*b$_{TTK}$ **(A-C)** and ab* and ab*$_{TTK}$ **(D-F)**. Data collected by Margaux Miller and Suzanna Bennett.

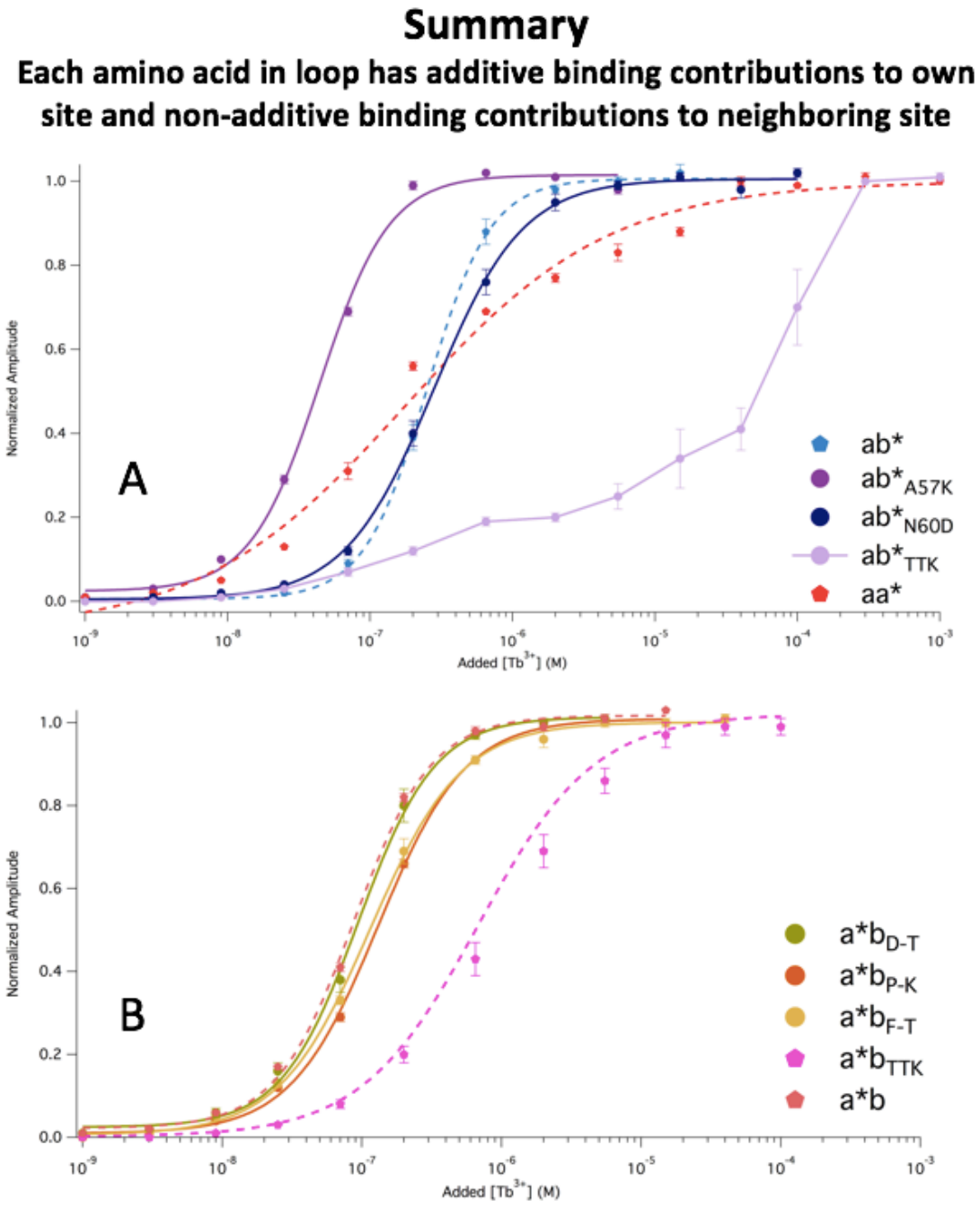

Figure 9. (A) The intermediate mutations each contribute to the shape of the aa* chimera (red, dashed) showing amino acids have independent, additive roles in governing cis-effects, yet the lack of any of the individual mutations in **(B)** to account for the change seen with them all together (a*b_{TTK}, pink, dashed) indicate dependence on combinations of amino acid changes in determining trans-effects. Each amino acid in the binding loop operates both to directly determine local binding in its own site, and as part of a collective to indirectly determine binding in the neighboring site. Data collected by Margaux Miller and Suzanna Bennett.