Domain-Specific Probes for Calcium Binding to Neural Cadherin

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DOMAIN-SPECIFIC PROBES FOR CALCIUM BINDING TO NEURAL CADHERIN

by
Charles Ryan Humphries

A thesis submitted to the faculty of the University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
May 2014

Approved by

_____________________________
Advisor: Dr. Susan Pedigo

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Reader: Dr. Bradley Jones

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Reader: Dr. Nathan Hammer
Dedication

I dedicate this thesis to my mother, Peggy Humphries, who has always shown me all of the love and support that a son could ask for. You have never settled for assuming that I was okay. You insist on sending me daily reminders that I am on your mind and that you are praying for me. When times are hard, I know that I can always talk to you. Thank you for all of the daily thoughts and prayers. I hope that I never take you for granted. I love you.

I would like to end this dedication with a poem, *A Mother’s Love* by Helen Steiner Rice.

A Mother’s love is something that no one can explain,
It is made of deep devotion and of sacrifice and pain,

It is endless and unselfish and enduring come what may
For nothing can destroy it or take that love away . . .

It is patient and forgiving when all others are forsaking,
And it never fails or falters even though the heart is breaking . . .

It believes beyond believing when the world around condemns,
And it glows with all the beauty of the rarest, brightest gems . . .

It is far beyond defining, it defies all explanation,
And it still remains a secret like the mysteries of creation . . .

A many splendored miracle man cannot understand
And another wondrous evidence of God’s tender guiding hand.
ACKNOWLEDGEMENTS

I would like to thank the members of Dr. Pedigo’s Lab who helped in the development of this thesis; specifically, Dr. Susan Pedigo, Dr. Nagamani Vunnam, Matthew Dukes, Xiaoyun Howard, and Jared Jungles.
Abstract

Cadherin is a major mediator of cell adhesion whose adhesive function is dependent upon calcium binding. However, the binding of calcium is not completely understood. The binding of calcium can be monitored using an abbreviated construct that contains only the first two extracellular domains (EC12) which includes two tryptophan residues, W2 and W113. Single tryptophan mutants were created which contain only W113 (W2A) and W2 (W113F). Fluorescence spectra were obtained of W2A, W113F, and WT in the presence and absence of calcium. Calcium titration data were collected for each protein. In both experiments, calcium binding increased the fluorescence signal of W113F and decreased the signal of W2. The apparent $K_d$ values from titration experiments showed a much lower affinity for calcium binding in the WT construct than in the single-tryptophan mutants. Simulations of calcium binding data address this apparent discrepancy. Two models were used to create simulations: an equal and independent model and an unequal and independent model. W2 signal, W113 signal, and the sum of the two, WT, were represented in the simulations. The effect of span, offset, and $K_d$ values were tested for the simulated W2 and W113 data to observe the effects on the summed data. Changes in offset of the two mutants had only a trivial effect. Span dictates the span of the sum, and also affects the $K_d$ value. When simulating an increasing W113 signal and decreasing W2 signal, the $K_d$ of the sum shifted to greater values. When both signals have the same direction, the sum is represented by an intermediate $K_d$. Therefore, the $K_d$ of the sum is dictated by the span, $K_d$, and direction of the signal of W2 and W113. This indicates that W2A and W113F are good reporters of the two separate classes of sites in EC12 constructs of N-cadherin.
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<table>
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<tr>
<td>A(#)</td>
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<tr>
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<td>Aspartate (Position)</td>
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<tr>
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<td>NCAD12 (W2 Replaced with A)</td>
</tr>
<tr>
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<td>$\Theta$</td>
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I. Motivation

As the major mediator of cell adhesion in adherens junctions, cadherins are critical for tissue formation and maintenance. Calcium binding is required for adhesive dimer formation, however very little is known about the linkage between the energetic or structural linkage between calcium binding and dimerization. We can monitor calcium binding using changes in spectral signals. Since there are three calcium ions that bind to the constructs we study, we must make assumptions about the signal change associated with specific binding events. It is difficult if not impossible to determine site-specific constants from such binding data. The studies herein use a combination of experimental and simulations to investigate several models for the intrinsic affinity and cooperativity of calcium binding. Based on comparison with experimental data these models will allow prediction of binding events that lead to adhesive dimer formation.
II. Review of Cadherin Structure and Function

A. Function

Adherens junctions are important cell-cell junctions that indirectly link the actin cytoskeleton between adjacent cells. Classical cadherin is the transmembrane protein in adherens junctions; cadherin’s extracellular domains from identical molecules on apposing cells interact in a direct protein-protein complex, while cytoplasmic domains interact with the actin cytoskeleton.[1] Induction of cadherin expression in development during mesenchymal-epithelial transitions (METs) drastically increases the adhesive properties of cells.[2]Reviewed in [3] Cadherin plays important roles in such events as embryogenesis, tissue morphogenesis, and wound healing.[4] Due to its importance in these key physiologically relevant processes, its structure-function relationship has been intensely studied in the ~ 30 years since they were first noted in the literature.

Cell-cell contacts between cells in developing tissues initiate cytoskeletal rearrangements that support the formation of adherens junctions. Cell-cell adhesion begins when actin-mediated lamellipodia and membrane ruffles from one cell contact protrusions from another cell.[5] This process is illustrated in Figure 2.1A, B, C. Following contact, cadherins migrate to and cluster at these contacting regions.[6] Cadherins initiate a cytoskeletal rearrangement in which thick actin clusters surround the cell-cell contact. This cytoskeletal rearrangement expands and stabilizes cell-cell contacts through introduction of additional points of contact following actin polymerization and physical pulling through acto-myosin tension.[5] Thus, while actin filaments influence the
distribution of cadherins at cellular contacts, clustering of cadherins at developing adherens junctions leads to rearrangement of the actin cytoskeleton. Cadherin’s interaction with the actin cytoskeleton is indirect and mediated by a number of cell-type dependent partners including several members of the catenin family. As illustrated in Figure 2.1D, the cytoplasmic domain of cadherin binds directly to p120 and β-catenin.

Cadherin is critical for creating tension in the cytoskeletal networks of interacting cells in a tissue. In embryogenesis, adhesion between cells greatly increases during compaction of the 8 cell stage. The driving forces of compaction may be similar to those of cell contact expansion (Figure 2.2). Membrane protrusions initiate contact and form bridges between cells. This initiation of cell-cell contact holds similar importance in wound healing and cell sorting. Wounding leaves holes in epithelial tissue which must be sealed by migrating epithelial sheets. In this process, lamellipodia contact and junctions are formed between cells. Contractile forces in the cytoskeletal networks act to seal the hole.

The role of dimerization of cadherins is regarded as important for cell adhesion, but what happens in the absence of this action? Several studies have attempted to answer this question through cadherin mutations and knockouts. Mutant constructs result in defective tissues and failure of cells to aggregate in culture. Reviewed in

As discussed previously, MET induces expression of cadherins and is involved with adherens junction formation in tissue morphogenesis and embryogenesis. Its opposite process, epithelial-mesenchymal transition (EMT), is associated with carcinogenesis. In carcinogenesis, a cadherin expression switch occurs which results in decreased expression of epithelial cadherin (ECAD) and increased expression of neural cadherin
Through mutations in mouse models, ECAD dysfunction has been linked to amplifying tumor progression. Normal expression of ECAD acts to suppress cell invasion and metastasis. The activation/deactivation pathway in which ECAD and NCAD are linked provides an additional suppression mechanism to ECAD expression. NCAD activation facilitates an activation pathway which leads to cleavage of ECAD by metalloproteinase-3 and further dissipation of ECAD.\textsuperscript{15-17} The distinct mechanism by which ECAD suppression initiates tumor progression is disputed, however.

Taken as a whole, the evidence points to the need for dynamics in adherens junctions that allow for changes in adherens junctions to promote normal functions such as tissue development and wound healing. In abnormal situations such as cancer, these dynamic characteristics lead to metastasis.\textsuperscript{14} Due to the importance of cadherin in solid tissue formation and maintenance, we are interested in the role of calcium in the formation of the adhesive dimer.
Figure 2.1 The Cellular Actions of Cadherin: A) This figure shows the actin protrusions which initiate cell-cell contact during the search phase. B) Following cell-cell contact via lamellipodia, cadherin is recruited to the site of contact. Buildup of cadherin leads to formation of cadherin puncta and actin bundles surround the contact. Mysin-mediated tension pulls inward and creates an intermediate structure known as the adhesion zipper. C) Following expansion of the contact, actin arcs provide stability on the edges of contact and the adhesion belt is formed. Taken from Reference[3]. D) Classical cadherin interacts directly with several cytoplasmic proteins. The interaction with β-catenin provides a mechanism by which cadherin can communicate with the actin cytoskeleton. β-catenin has multiple possible binding partners including vinculin which then binds α-catenin.[11] α-catenin binds and bundles actin filaments.[12] The specifics of the linkage from β-catenin to actin are unknown. This linkage between cadherins and the actin cytoskeleton provides a means for tissue-scale responses. Taken from Reference[13].
**Figure 2.2** Commonalities Between Cell-Cell Junction Formation and Wound Healing:

A) Pictured is the process of wound healing of a hole in epithelial tissue. (N) represents nuclei of individual cells. Upon contact, cadherin is recruited to the site of contact and junctions are formed. This interaction leads to cytoskeletal rearrangements and promotes sealing of the hole. Boxes surround the area of commonality between cell-cell junction formation (B) and wound healing (A). Included within the boxes are the adherins junctions and the actin clusters which stabilize the cell-cell contact and provide contractile forces between cells. Taken from Reference[3].
B. Structure

The importance of cadherins in processes involving cell-cell adhesion has promoted much research surrounding this class of cell adhesion molecules. Structural and domain dissection experiments have identified three main regions in cadherins: as a single conserved cytoplasmic domain which reacts with catenins and the actin cytoskeleton, a single-pass transmembrane domain, and a chain of extracellular cadherin-like domains (ECs). Each EC is composed of seven anti-parallel strands of two β-sheets in a Greek-key motif that are approximately 110 amino acids each.\(^{[18]}\) In this thesis, we will focus on type 1 classical cadherins which contain an extracellular portion of 5 ECs (EC1-5) (Pictured in Figure 2.3). The most commonly studied type 1 classical cadherins are epithelial cadherin (ECAD) and neural cadherin (NCAD). Adherens junctions require direct protein-protein interactions between identical cadherins from two juxtaposing adherent cells.\(^{[6]}\)

Calcium is required for cadherin-mediated cell-cell adhesion. Classical cadherins contain twelve calcium binding sites, three between each successive EC modular domain. The calcium dependence of cadherins for dimerization and cell-cell adhesion has been demonstrated through numerous cell aggregation studies.\(^{[19]}\) The binding of calcium causes a global structural change in the EC region which leads to organization into a curved shape, decreases the susceptibility of cadherin to proteolysis, allows the domains of cadherin to act as a cooperative unit and creates strain in the monomeric structure that is relieved upon formation of dimer.\(^{[20]}\)

Although the cooperative unit is required for the complete function of cadherin, recent studies have shown that a unit of EC1 and EC2, EC12, is useful for studying
Figure 2.3 Ribbon Drawing of the EC Domain of E-Cadherin. The 5 modular domains of E-cadherin are shown as labeled EC1 to EC5 in two images that differ by 90° in rotation. EC1 is the most distal domain and the site of the adhesion interface. Calcium ions (green) are shown at the interface between adjacent EC-modules. Possible glycosylation sites are shown in red. The insert to the right is an enlargement of the adhesive dimer interface which is a strand-swapped structure. The side chain for W2 is shown interacting with the partner protomer across the dimer interface. Taken from Reference[20].
calcium binding and dimerization. This two domain construct must be EC12, specifically, due to the importance of EC1 in dimerization through \textit{trans} \(\beta\)-strand swapping \cite{21,22} An N-terminal tryptophan residue in EC1, W2, is responsible for strand-swapped dimer formation. In a cadherin monomer, W2 is docked within a hydrophobic pocket near the N-terminus of its own EC1. The binding of calcium ions between EC1 and EC2 causes a conformational change which undocks W2 from its own hydrophobic pocket. This allows W2 to dock into a partner cadherin’s hydrophobic pocket, leading to strand-swapped dimerization \cite{22}

Due to the similarities of the environment of W2 in closed monomer and dimer states, dimerization is a low affinity event. The existence of a self-docking site promotes competition between the monomeric and dimeric species. Therefore, dimerization must be induced by either destabilizing monomer or by stabilizing the formation of dimer. \cite{23} Calcium-induced destabilization of monomer through undocking of W2 is one way that dimerization is promoted. Dimer formation is also stabilized through formation of a salt bridge between a glutamic acid residue, E89, and the N-terminus aspartic acid residue, D1, of the partner EC1. \cite{24} This noncovalent interaction has been shown to greatly decrease the energy required for dimerization.

Another potential driving force toward dimerization is formation of the X-dimer, a second dimer form demonstrated through observation of crystal structures and a few recent functional studies (\textit{Figure 2.4}). \cite{25} In the X-dimer, the \(\beta\)A-strands of partner molecules, the strand which includes W2, are juxtaposed, suggesting a role as a close-encounter complex. Data from SPR, size-exclusion chromatography, and velocity AUC experiments suggest that the formation of X-dimer increases kinetics of the formation of
the strand-swapped dimer by placing the interacting protomers in a face-to-face orientation so that the \( \beta A \)-strands can swap. The structural design and experimental data surrounding the X-dimer suggests that it is an intermediate between monomer and strand-swapped dimer that functions as a transition state promoting rapid exchange between monomer and dimer when calcium is present.\textsuperscript{[25]} The requirement for calcium is a defining characteristic for adherens junction formation. In the next section, calcium binding will be discussed.

\textbf{Figure 2.4} Ribbon Drawings That Represent Structures for the Monomeric Structure, the X-Dimer Structure and the Strand-Swapped Structure. The binding of calcium to the monomer creates strain that is relieved upon formation of the strand-swapped dimer. The transition state between monomer and dimer is too high to be overcome with the formation of a transient intermediate that juxtaposes the strand-swapped interface so the \( \beta A \)-strands can exchange between protomers. Taken from Reference \textsuperscript{[25]}.  


III. Calcium Binding

Calcium binding is one topic which is not completely understood, but is pivotal for cadherin function in cell-cell adhesion through homophilic dimerization. Calcium binds at the interdomain interface between each successive modular EC-domain. The following discussion is focused on the 3 calcium ions that bind at the EC1-EC2 interface since the EC1-EC2 construct is the minimal functional subunit and is under study here. Critical residues for calcium binding to the three sites in EC12 are highlighted in Figure 3.1.

Sites comprise clusters of aspartate and glutamate residues, and are numbered sequentially as established in the original structure of the EC1-EC2 construct of E-cadherin.[26] Calcium ions are in very close proximity (≤7Å) compared to EF-hand type calcium binding sites (≈11Å). IN fact, there are several amino acids whose side chain oxygens are shared between two sites. The 3 amino acids shared between sites 1 and 2 indicate their close connection (≈4Å separation).

Notice in Figure 3.1 that the residues comprising site 1 are all located in EC1 with D103 as an exception which resides in the interdomain linker region. Residues comprising site 2 are in EC1, the interdomain linker, and EC2. Site 3 is comprised primarily of residues in EC2 with a few contributions from the interdomain linker region. Thus, the sites become increasingly buried in the interdomain region as we proceed from site 1 to sites 2 and 3.
Site-specific mutations of D134, D136, and D103 in neural cadherin EC12 (NCAD12) were induced in order to identify the importance of these residues and their role in calcium binding within the cadherin protein. The proteins were analyzed using UV-vis spectroscopy. The spectra of the mutants were very similar to the UV spectra of wild type cadherin. This evidence signifies an identical or slightly changed environment of the spectroscopic probes in the mutant and wild type proteins. Shared secondary structures were confirmed by using Circular dichroism (CD) spectroscopy. The apo states of mutant and wild type proteins were also shown to share similar stabilities.[22]

Calcium titrations, partnered with CD and UV spectra, were used to evaluate the effects on calcium binding affinity from each mutation. In the absence of structural change to the apo state, these mutations were still able to decrease the affinity for calcium binding. Therefore, each of these sites must monitor calcium binding to at least one of the calcium-binding sites. Mutation of D134, a critical residue for calcium binding to site 3, resulted in failure to bind to all three sites. Mutation of D136, the linkage between site 3 and site 2, also resulted in impaired binding to all three sites. Mutation of D103, which links site 2 and site 1, resulted in full occupancy with a slight decrease in binding affinity. Although full occupancy was maintained, equilibrium dialysis and assembly studies showed that the D103 linkage is essential for dimerization.[22]

In summary, studies of calcium binding to cadherin show that binding occurs by a precise order of events. Site 3 must bind first, or sites 1 and 2 will not bind, and dimerization will not occur. Secondly, sites 1 and 2 are in close proximity and they are connected by 3 common residues. This is a surprisingly close interaction between these
Figure 3.1 Summary of Calcium Binding to N-Cadherin: (A) Ribbon drawing of EC1-EC2 of E-cadherin to illustrate the “minimal functional unit for folding, calcium binding and dimerization. Calcium ions are shown in yellow. Helices are colored A through G as red, orange, yellow, green, blue, cyan and purple. (B) Illustration of the amino acid residues in cadherin that comprise each binding site. (C) A table showing the participant amino acids in each site. Note that Site 1 comprises residues from EC1 and the linker region, site 2 comprises residues from EC1, linker region and EC2, and site 3 comprises residues from the linker and EC2. The 3 bidentate residues shared by sites 1 and 2 are E11, D69 and D103. Taken from Reference [22].
two sites, such that one might expect calcium binding to these two sites to be highly cooperative. Finally, the linkage between sites 1 and 2 is required to generate the strain in the monomer that drives dimer formation.
IV. Review of General Concepts

Fluorescence is useful as a tool in cadherin studies due to the presence of intrinsic tryptophan fluorophores. In our EC12 construct, we are provided two spectroscopic probes, W2 and W113. W2 resides near the N-terminus of EC1, while W113 is within EC2. As will be discussed later, the two residues have distinct spectral signals and may prove useful for determining site specific affinities.

In proteins, the aromatic amino acids phenylalanine, tyrosine, and tryptophan are responsible for fluorescence and can each be excluded from emission spectra by choosing specific absorption wavelengths. The wavelength 280 nm is above the absorption maximum wavelength for phenylalanine, therefore fluorescence spectra produced with absorption wavelengths above 280 nm are absent of phenylalanine fluorescence. Tyrosine fluorescence can also be excluded when working at wavelengths over 295 nm. Tryptophan dominates fluorescent spectra due to a higher absorption wavelength and a greater extinction coefficient. By using an optimal absorption wavelength between 295-305 nm, tryptophan fluorescence can be the sole contributor to fluorescence in fluorescence spectra. Tryptophan fluorescence occurs through overlap of two $\pi\pi^*$ transitions to two nearly isoenergetic states, $^1L_a$ and $^1L_b$, which display distinct spectra. The emission spectra for tryptophan peaks around 340 nm.[27]

The process of fluorescence is illustrated by a Jabłoński diagram (Figure 4.1). The process of fluorescence begins when electrons in are promoted to an excited electron state which rapidly decays nonradiatively to the lowest vibrational state of the first
excited state. Fluorescence excitation operates in singlet states, which refers to the pairing of each electron to an electron of opposite spin which sum to an angular momentum of zero. Fluorescence allows for rapid conversion from the excited state to the ground state which is achieved through the emission of a photon. Fluorescence emission energies are less than the absorbance energies and the difference in the higher energy excitation and lower energy emission is called a Stokes’ shift.[27]

Stokes’ shifts refer to a loss of energy which can be explained by several occurrences following excitation. Whenever an electron is excited to the S2 phase, it commonly undergoes internal conversion and relaxes to the lowest vibrational level of S1. Electrons excited to higher vibrational levels of S1 also undergo an identical process known as vibrational relaxation. Another contributing factor, decay to higher vibrational levels of S0, results in the greatest Stokes’ shift and additional loss of energy.[27]

Individual fluorophores or molecules may exhibit larger Stokes’ shifts due to solvent effects, excited-state reactions, complex formation, and energy transfer. In the case of tryptophan, changes in environment and solvent polarity can lead to shifts in emission wavelength. This phenomenon usually occurs in fluorophores which exhibit a charge separation in the excited state, but in the case of tryptophan, it is due to the existence of the 1L_a and 1L_b states. The overlap of these two states complicates the long-wavelength absorption (240-300 nm) as compared to a normal fluorophore which is excited into S1. 1L_a and 1L_b have different dipole moments which dictate which excited state is the lowest energy in a particular environment. The lowest energy excited state is responsible for emission and thus dictates the characteristics of emission. Polar solvents stabilize the excited state dipole, thereby compressing the energetic difference between
Figure 4.1 Jabłoński Diagram: The process of fluorescence is illustrated by depicting energy changes as arrows. The singlet levels of ground state, first excited state, and second excited state are represented by $S_0$, $S_1$, and $S_2$, respectively. Electronic energy levels are further divided into vibrational energy levels corresponding to 0, 1, 2, etc. Energy transitions between states are depicted as vertical lines to portray the instantaneous of the processes (on the order of $10^{-15}$s). First, energy must be absorbed to move electrons into excited states. Following absorption, internal conversions and vibrational relaxation may take place to bring the electrons into the lowest vibrational level of $S_1$. Return to $S_0$ results in the release of a photon and fluorescence. Adapted from Reference [27].
the $S_1$ and $S_0$ states. This results in a decrease in the energy difference between excitation and emission wavelengths. Blue-shifts (shift to shorter wavelengths), higher energy transitions, correspond to a tryptophan residue that is buried in the hydrophobic core of the protein. Red shifts (shift to longer wavelengths), lower energy transitions, correspond to a tryptophan residue that is exposed to the aqueous environment in which most soluble, globular proteins are exposed, including cadherin.\textsuperscript{[27]}

In our EC12 construct, tryptophans provide intrinsic properties for analysis. The three calcium-binding sites of our EC12 construct are within each of the domains. Sites 1 and 2 are within domain 1 along with the W2 spectroscopic probe. Site 3 is in domain 2 with the W113 probe. It is the goal of this thesis to approach the idea of calcium-binding site specific monitoring by the fluorescence of W2 (buried) and W113 (40% exposed).
V. Fluorescence Spectra

In order to investigate the unique response of W113 and W2 to the binding of calcium, we created two single-tryptophan mutants, W2A and W113F. The fluorescence signal from W2A is solely from W113, and from W113F is solely from W2. Wild type (WT) includes signals from both W2 and W113. Fluorescence spectra were taken for all three EC12 constructs as a function of calcium (Apo and 1mM calcium added). The spectra can be seen in Figure 5.1. The concentration of NCAD12 was low, 5µM, in order to maximize signal to noise while discouraging association of dimer. The calcium binding sites should be >90% saturated at 1mM calcium.[28] The pH was 7.4.

Fluorescence emission maxima for WT, W2A, and W113F were observed at 331 nm, 336 nm, and 324 nm, respectively. The fluorescence spectra from W2A are red-shifted relative to W113F. This indicates that W113, the fluorescent probe in W2A is more exposed to solvent than W2, the only fluorescent probe in W113F. The WT spectra indicate contributions from both W2 and W113. The wavelength peak fluorescence intensity does not vary with addition of calcium, indicating that calcium binding does not change the environment of tryptophans. The (+) calcium state increases the fluorescence intensity of W2A and decreases the fluorescence intensity of W113F. Therefore, while the signal of W113 (from W2A) is strengthened in the presence of calcium and the signal from W2 (from W113F) is weakened. The WT spectrum also shows an increase in fluorescence intensity.
It is important to note that these spectral results concerning the exposure of W2 and W113 are consistent with the solvent exposure of the tryptophans in structural studies. Using GetArea, the solvent accessibility of W2 and W113 were determined to be 10% and 40% respectively. Thus, based on solvent accessibility, one would expect W2 to yield a blue shifted fluorescence emission compared to W113, which was observed. Also, it is important to note that when proteins were unfolded in Guanidine HCl, they yielded identical F1 emission spectra with a maximum wavelength of 345 nm, indicating a uniform and increased exposure to solvent when unfolded. The sums of (+/-) calcium conditions for W2A and W113F were also calculated. Interestingly, the sum of the spectra for W2A and W113F closely resemble the plots for WT NCAD12. The information gained from the (+/-) calcium spectra provided insight into possible correlations between calcium binding to tryptophan and fluorescence. Models were created to investigate these correlations.
Figure 5.1 Fluorescence Spectra as a Function of Calcium. Fluorescence spectra of the NCAD12 constructs are shown in the absence (open) and presence (solid) of calcium. Comparison of WT (black), W2A (blue), and W113F (green). The sum of the signal from W2A and W113F is shown in presence (pink dashed) and absence (pink solid) of calcium. The W2A and W113F mutations prompted little change in proteins. The summing of W2A and W113F signals to the WT signal provides support of negligible effects to overall protein conformation. This data led support for a subsequent experiment to study the binding of calcium in WT and the two single-tryptophan mutants.
VI. Results

A. Titration Data

Following the results from the fluorescence spectra study (Figure 5.1), we saw potential for the same comparison in a calcium titration study. The two single-tryptophan mutants and wild-type protein were titrated with calcium by addition of small volumes of a series of concentrated calcium solutions at pH 7.4. To yield data over \( \approx 5 \) orders of magnitude in calcium concentration, calcium stocks at 1mM, 10mM, 100mM, and 1M were used. After each calcium addition, a FL emission spectrum was acquired. Excitation wavelength was 295 nm in order to only excite tryptophans. Raw data from representative titrations of W2A, W113F, and WT protein are shown in Figure 6.1.

From the data in Figure 5.1, the effects of calcium binding were demonstrated for both W2 and W113. From this data it was seen that addition of calcium increases fluorescence intensity for W2A which only has a W113 fluorescent signal. Calcium binding decreases the fluorescence intensity for W113F which only has a W2 fluorescent signal. Therefore, the fluorescence spectra of titration data for W2 should be decreasing and the spectra for W113 should be increasing. We are also assuming that the spectra of the single-tryptophan mutants are summable to give the WT spectra. This is because WT contains both W2 and W113 as spectroscopic probes.

After further examination of Figure 5.1, it is seen that the WT spectra follows the trend of W2A with increasing fluorescence intensity in conjunction with the binding of
calcium. As W2A and W113F show opposite trends, the increasing trend for WT is due to a larger span of W2A as compared to W113F.

The spectra for the titration experiment were taken at 331 nm for WT, W2A, and W113F. Data describing the fits of Equation 1 to each titration curve can be found in Table 6.1. In order to understand the acquired data, models were created to examine the effects of specific changes to $K_d$, span, and offset. The titration data was fitted to an equal and independent site model based off of the Adair equation:

$$\theta = \frac{K[X]}{1 + K[X]}.$$  (1)

The variable $n$ represents the number of sites, $K$ represents the $K_a$ of calcium to specific sites, and $[X]$ represents calcium concentration. $\theta$ is representative of the fraction of occupied binding sites. $\theta$ was scaled to fluorescence intensity using the equation:

$$Fl \text{ intensity} = \theta \ast span + offset.$$  (2)

Span and offset act as scaling factors. Data was plotted to represent specific calcium-binding site conditions. The graphical models are shown in Figures 6.2 and 6.3.
Figure 6.1 Experimental Determination of Calcium Binding Affinity to W2A, W113F, and WT NCAD12. Fluorescence signal (cps) was monitored at 331 nm for all 3 proteins as a function of total calcium. Data points are shown. Solid line is based on a fit of the data to an equal and independent site model (Equation 1). Total calcium is approximately equal to free calcium.
B. Introduction to Models

Models fitted to the same equations as the titration data were created in order to understand the experimental data. In our EC12 construct there are two spectroscopic probes, W2 and W113 and 3 calcium binding sites between EC1 and EC2 which exhibit sequential binding in the order of site 3, then site 2, then site 1. Sites 1 and 2 are within EC1 and site 3 is within EC2. We hypothesize that W2 can act as a fluorescent monitor of calcium binding to sites 1 and 2 and W113 can monitor binding to site 3. Sites 1 and 2 are linked by three amino acids: E11, E69, and D103. These linkages, as well as the close proximity of the sites, provide hints toward cooperativity between the two sites. D136 links sites 2 and 3, but the cooperativity between sites 1 and 2 is believed to be on a much greater scale. Therefore, for simplicity, models were created to show a theoretical system which includes two spectroscopic probes and two calcium binding sites. For the sake of the models, sites 1 and 2 were combined due to their suspected cooperativity. Site 3 is treated as its own individual independent site.

Based on the preceding discussion we propose a model as follows:

- Site 3 binds calcium first,
- Binding to site 3 is relatively independent of binding to sites 1 and 2,
- The primary conformational change from binding to site 3 occurs in EC2,
- Binding to sites 1 and 2 is cooperative,
- The primary conformational change from binding to sites 1 and 2 occurs in EC1.

Although calcium binding to cadherin is likely cooperative, the simulations presented in the body of this document are created from the equal and independent site
model and consider two classes of sites. We take advantage of the location of a single tryptophan in each of the two domains, leading to “site-specific” probes of ligand binding. The concept is that the signal from WT protein is the sum of the signals from the two single-tryptophan mutants. Moreover, we expect that the simulations will model the free energies or affinities of binding to each class of site provided that the mutations do not impact calcium binding affinities.

The simulations of the equal and independent site model were constructed based on the following calculations. Calcium concentration was increased by 1.2 times for each data point, starting with a concentration of $1.0 \times 10^{-7}$M. Calcium concentration was converted to the logarithmic scale on the graph. The y-axis is representative of fluorescence intensity and was calculated by using Equations (1) and (2).
C. Discussion of Data and Simulations

The data from the titration study can be found in Table 6.1. When comparing the data, it is seen that the signal for W113F begins at a much smaller intensity, 155, while W2A and WT signals begin at much higher intensities (301 and 307, respectively). The initial signals are known as offset. The spans of each signal were calculated by

\[ \text{final FL intensity } - \text{initial FL intensity (offset)} = \text{span}. \] (3)

The spans for W2A and WT are positive, while the span for W113F is negative. Interestingly, these values are summable in theory and in practice. The

\[ \text{Span (W2A)} + \text{Span (W113F)} \approx \text{Span (WT)}. \] (4)

In the data provided by the calcium titration, the offsets of W2A and W113F were not summable to equal the offset of WT. The sum instead provided a much higher offset value than the experimental value for WT. From examinations of the \( K_d \) values, it is seen that they show an increasing trend from W113F< W2A< WT. Smaller \( K_d \) values are representative of higher affinity for ligand binding. Why are the apparent affinities for W2A and W113F higher than that of WT? We would expect to see a binding affinity for WT that is the weighted sum of binding affinity of each single-tryptophan mutant.

There are several possible molecular explanations for why mutants would have apparently higher calcium binding than WT. For W2A, one possibility can be attributed to the fact that the W2 residue in WT protein induces strain in EC1 upon calcium binding. If W2 is absent (as it is in W2A), then we would expect slightly higher affinity binding of calcium because the free energy of calcium is reduced in binding to WT, in part, to induced strain between W2 and the hydrophobic pocket. Alternatively, if the mutations change the linkage of calcium binding to dimerization, it might impact
Table 6.1 Data from the Titration of NCAD12 Constructs with Calcium. Listed are endpoints, span, and $K_d$ values for W2A, W113F, and WT protein. Data was taken from the calcium titrations shown in Figure 6.1. These data provides insight into the ability to sum data from titration curves. The sum of span (W2A) plus span (W113F) provides an almost identical value to the span of WT. Lower $K_d$ values are representative of the higher binding affinity. The constructs were ranked for binding affinity as follows: WT<W2A<W113F.

<table>
<thead>
<tr>
<th>EC12 Construct:</th>
<th>W2A</th>
<th>W113F</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endpoints (x10^3)</td>
<td>301→355</td>
<td>155→132</td>
<td>307→339</td>
</tr>
<tr>
<td>Span:(x10^3)</td>
<td>+54</td>
<td>-23</td>
<td>+32</td>
</tr>
<tr>
<td>$K_d$</td>
<td>68.1µM</td>
<td>33.5µM</td>
<td>143µM</td>
</tr>
</tbody>
</table>
estimates of $K_d$ for calcium.

Inspection of the $K_d$ values presents a conundrum. How can the binding affinities of the single-tryptophan mutants be greater than WT? Why would the sum of 2 higher affinity events at 68-34$\mu$M in the single-tryptophan mutants equal a lower affinity binding event in WT? This doesn’t make sense! Simulations yield insight into this problem.

Our simulations began with the equal and independent model, a model to simulate the most simplistic conditions for calcium titration data. Equal refers to equivalent $K_d$ values. Independent refers to the absence of cooperativity between sites in a class with each class being represented by the signals of W2 and W113. A third data set was made to represent WT signal and is analytically the sum of W2 and W113 signals. Consecutive models were made by altering offset and span conditions. Generally, the signal from W113 increases over the course of the titration while the signal from W2 decreases. From these simulations, we were able to view the effects of changes in span and offset through comparison of midpoints (apparent $K_d$).

In the first set of simulations the simulated data for W2 and W113 assumed equal affinity and independent binding in each of the two classes of sites. Span and offset parameters were varied to test their effect on the apparent $K_d$ of the summed data (apparent WT). From comparison of the top two simulations in Figure 6.2, it is seen that equal but opposite spans sum to an apparent data set with a span of 0 indicating that there would be no fluorescence signal from WT if the signals from W2 and W113 had identical affinities and changes by the same amount, but in opposite directions. Offset differences in the simulated data sets have no apparent effect. The third simulation of Figure 6.2
provides insight into the effect of changes in span. Changes in span of the two single-
tryptophan mutants yields a “sum” isotherm with a decreased span but identical value for
$K_d$. The bottom graph again shows that offset makes no difference in the apparent $K_d$ for
the “sum” data set. In summary, if the two classes of binding events are equal and
independent, and change in opposite directions with different spans, then they yield a
summed isotherm that has an identical $K_d$ value. For our purposes here, this implies that
the two classes of sites in WT NCAD12 are unequal affinity.
Figure 6.2 Equal and Independent Model. These simulations were created using Equations 1 and 2. The simulations were made to mimic the experimental trends of W2A and W113F. The W2A signal, which only contains the W113 spectroscopic probe, is shown in blue and has a positive span. The W113F signal, which only contains the W2A spectroscopic probe, is shown in red and has a negative span. The sum of these two data sets is pictured in green. The sum of W2 and W113 signals is representative of WT protein. The $K_a$ value is held constant at 9000 for both the W2 and W113 signals. This model is representative of independent sites that show no cooperativity. Conditions were set for $=$span with $=offset$, $=span$ with $\neq offset$, $\neq span$ with $=offset$, and $\neq span$ with $\neq offset$. The effects of changes in span and offset can be observed in these four simulations. $K_a$ values for the sum were calculated for each condition.
Moving on from the simplest model, the equal and independent model, we created an unequal and independent model. Unequal corresponds to unequal $K_d$ values for the single-tryptophan mutants and independent refers to the lack of cooperativity between sites in each of the two classes of sites. For these models, $K_d$ values of 111 µM and 40 µM were used to represent W113 signal and W2 signal, respectively. This model is a better representation of what happens in our system, because of the differing $K_d$ values resolved in the calcium titrations of W2A and W113F.

The top two simulations (Figure 6.3) illustrate that the difference in the midpoints of the simulated curves for the two single tryptophan mutants leads to a dip in the “sum” isotherm, and that the offset value makes no difference. $K_d$ cannot be calculated. The bottom two simulations of Figure 6.3 provided very interesting insights into the experimental data. By differing span, a $K_d$ value can be calculated for the sum of W2 and W113 signals. This value for the apparent $K_d$ of the sum, 227 µM, is much higher than the $K_d$ values used for W2 and W113 signals (40 µM and 111 µM, respectively). Therefore, WT is shown to have a much lower apparent affinity for calcium than W2A and W113F. Thus, these simulations illustrate the same phenomenon that occurred in the titration data in Figure 6.1. Why then, does this push to higher $K_d$ values occur?
Figure 6.3 Unequal and Independent Model. These simulations were created using Equations 1 and 2. The models were made to mimic the experimental trends of W2A and W113F. The W2A signal, which only contains the W113 spectroscopic probe, is shown in blue and has a positive span. The W113F signal, which only contains the W2 spectroscopic probe, is shown in red and has a negative span. The sum of these two data sets is pictured in green. The sum of W2 and W113 signals is representative of WT protein.

The difference between these models and Figure 6.2 is that the $K_d$ of the W2 signal is set at 40µM. The $K_d$ for W113 remains at 111 µM. This model is representative of independent sites that show no cooperativity.

Conditions were set for =span with =offset, =span with ≠offset, ≠span with =offset, and ≠span with ≠offset. The effects of changes in span and offset, in the presence of unequal $K_d$ values can be observed in these four graphs. $K_d$ values for the sum were calculated for each condition.
In order to investigate the origin of the push of the sum $K_d$, additional simulations were created with differing conditions. These additional simulations can be seen in Figure 6.4. The top two simulations are a comparison of two ≠span and ≠offset unequal and independent simulations. Offset was kept constant between the two simulations, due to its lack of effect on $K_d$ that was represented in Figures 6.2 and 6.3. In the top simulation of Figure 6.4, a span of -0.5 was used for W2 signal and in the second simulation of Figure 6.4, a span of -0.3 was used. Changes in span were shown to have great effects on the apparent $K_d$ of the sum. The push to higher $K_d$ of the sum was consistent in these two simulations. These two simulations showed that the magnitude of the $K_d$ push of the sum is dictated by the magnitudes of the spans of the signals of the single-tryptophan mutants.

The bottom two simulations in Figure 6.4 were created to address the question as to why the $K_d$ of the sum is pushed to higher values. In the spectra of our NCAD12 constructs, one signal is positive (W113) and one signal is negative (W2). The effects of differing spans were shown in the top two simulations of Figure 6.4. In the bottom two simulations of Figure 6.4, the directionality of the single-tryptophan mutants was addressed. Instead of one negative signal and one positive signal, both signals were made positive. The third figure shows an unequal and independent model of positive signals and the bottom figure shows an equal and independent model of positive signals. In the unequal and independent simulation, the $K_d$ value was equal to 6.67E-5, an intermediate between the $K_d$ values of W2 and W113 as portrayed in Figure 6.4. The summing of two positive signals in the equal and independent model had no affect on apparent $K_d$. The $K_d$ of the sum was equal to the $K_d$ of both single-tryptophan mutants.
Figure 6.4 Additional Simulations to Investigate the Push to Higher $K_d$ Values of the Sum of W2 and W113 Signals. These simulations were created using Equations 1 and 2. The top two simulations were made to mimic the experimental trends of W2A and W113F. The W2A signal, which only contains the W113 spectroscopic probe, is shown in blue. The W113F signal, which only contains the W2 spectroscopic probe, is shown in red. The sum of W2 and W113 signals is pictured in green. The sum of W2 and W113 signals is representative of WT protein. In the top 3 simulations, $K_a$ values of 9000 and 25000 were used to represent the $K_a$ values of W2A and W113F, respectively. In the bottom simulation, both W2 and W113 are represented by a $K_a$ of 9000. The top two graphs show the effects of differing spans in a simulation of W2 and W113 of different offsets. The bottom two simulations show the changes which occur under unequal and independent and equal and independent conditions when both signals (W2 and W113) have positive spans. $K_d$ values for the sum were calculated for each condition.
From the simulations in Figure 6.4, some important realizations were made. From the top two simulations, it was shown that the magnitude of the shift in $K_d$ of the sum is dictated by the values of the spans of the single-tryptophan mutants. As for the reason behind the shift, this issue is addressed in the third simulation. Whenever the signals of both single-tryptophan mutants are positive, an intermediate apparent $K_d$ is obtained for the sum. However, whenever one signal is positive and one is negative, a push to higher $K_d$ for the sum is observed. The comparison of these two conditions shows that the reason behind the push to higher $K_d$ lies in addition of a positive signal and a negative signal.
VII. Conclusion

Through comparison of all of the simulations in Figures 6.2, 6.3, and 6.4, several conclusions can be made. First, as demonstrated in many circumstances, offset had no effect on $K_d$ value of the sum in both the equal and independent model and the unequal and independent model. The determinants of the apparent $K_d$ of the sum were found to be $K_d$ values of W2 and W113, directions of the signals of W2 and W113, and spans of W2 and W113.

The effects of $K_d$ values were shown in comparison of the equal and independent model (Figure 6.2) and the unequal and independent model (Figure 6.3). $K_d$ of the sum in the equal and independent model remained constant (at the same value of W2 and W113) no matter what conditions were present. Therefore, in order to have a differing $K_d$ value from the single-tryptophan mutants, the $K_d$ of the W2 signal must be different than the $K_d$ of the W113 signal.

When there is a differing $K_d$ value between W2 and W113, the $K_d$ of the sum will have a different value than either of the mutants. The placement of this value; however, is dependent upon the direction of the signals of W2 and W113. If both signals are positive, the $K_d$ of the sum is an intermediate value between the $K_d$ values of the two signals. However, whenever one signal is positive and one signal is negative (as is the case of W113 and W2), the $K_d$ of the sum is shifted to a higher value than both W2 and W113.

The third variable that affects the $K_d$ of the sum of W2 and W113 signals is span. The span dictates to what degree the $K_d$ of the sum is increased by. Greater positive span result in higher apparent $K_d$ of the sum.
In order to test the validity of the unequal and independent model, values for span, offset, and \(K_d\) were taken from Table 6.1 to be used as data points. The simulation is shown in Figure 7.1. The previous statements regarding the effects of changes in \(K_d\) values, direction, and span were all exemplified in this simulation. One positive signal and one negative signal led to a \(K_d\) of the sum that was greater than both the signal for W2 and W113. The magnitude of this increase, however, was not as great as shown in other models. We believe that this slight increase may be due to the small spans exhibited by W2 and W113.

While many conclusions have been made through the use of the simulations, one thing is certain. The unequal and independent model is not sufficient for describing the actual event of calcium binding in NCAD12. Offset for the sum was at \(-4.55 \times 10^5\) for the simulations and was only \(3.07 \times 10^5\) in the titration study. The \(K_d\) values also showed differences. Although the trend of a shift to higher values was exhibited, the simulations provided a \(K_d\) value of \(107\mu\text{M}\) for the sum while the titration data provided a value of \(143\mu\text{M}\). The Span, however, was very similar. The simulations showed a Span of \(3.1 \times 10^4\) for the sum while the titration data provided a value of \(3.2 \times 10^4\). This provides insight that the unequal and independent model may be sufficient in predicting span, but not offset or \(K_d\). The inconsistencies between the unequal and independent model and the experimental data can be explained by the simplicity of the unequal and independent model. It does not take into consideration cooperativity, which is believed to be very apparent in the calcium binding process. This is the next step for the single-site models. Simulations with an unequal and cooperative model may be useful in describing the cooperativity between sites. Cooperativity, however, is not a simple variable to describe.
Figure 7.1 Unequal and Independent Model Using Experimental Values from the Titration Study. Values for offset, $K_d$, and span were taken from Table 6.1 in order to directly compare experimental data and values expressed in the simulations. The signal for W113 was positive (blue line) and the signal for W2 was negative (red line). The sum of the two signals is represented by the green line. Apparent $K_d$ value for the sum was calculated with the simulation data.
accurately. A system is not merely cooperative or independent. There are different levels of cooperativity. For example, we expect sites 1 and 2 to be very cooperative due to their connections through critical residues and proximity. Site 3 is thought to be cooperative with site 2 due to linkages by critical residues, but is expected to be less cooperative than sites 1 and 2. Models have been created for the unequal and cooperative model, but their usefulness has not been tested.

In other considerations, the proposed sequential binding model is not supported by the results in this thesis. The lower $K_d$ value of W2 represents a higher binding affinity for calcium than W113. Due to the domain-specificity of these two probes, it is obvious that binding to site 3 cannot occur before binding to sites 1 and 2.
BIBLIOGRAPHY


