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Calcium Binding Affinity Properties of Neural Cadherin Domains 1 and 2

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Abstract

Thomas Thayer Wood: Calcium Binding Affinity Properties of NCAD12

(Under the direction of Susan Pedigo)

Cadherins are a family of cell adhesion receptors that are crucial for binding of mutual vertebrate cells. Cadherins are calcium-dependent, homophilic, cell-adhesive molecules that are found in varying types of cell-cell contacts. Cell-cell contacts that contain cadherins include adherens junctions, desmosomes, and synapses. Cadherins play an important role in regulating maintenance of adult tissue structural design and integrity, tissue morphogenesis and synaptogenesis, and embryogenesis. Cadherins have an intracellular, cytoplasmic, region, a transmembrane region, and an extracellular region where calcium binding occurs. In classical cadherins, the extracellular region has five modular β-barrel domains and calcium binds to the interfaces between domains. Much effort has gone into characterizing the mechanism for cadherin cell adhesion, and it is widely accepted that the first two extracellular domains, closest to the N-terminus, are the minimal requirement for calcium binding and cell adhesion. Literature has suggested that the binding of calcium provokes conformational changes in the extracellular domain of classical cadherins by opening strand βA and exposing an essential tryptophan in the second position, W2, which then leads to cell-cell adhesion. However, these are the first solution studies of neural cadherin that have been attempted. Although calcium binding is known to be required in cadherin mediated

cell-cell adhesion, the mechanisms behind calcium binding have been extremely hard to characterize.

The purpose of these experiments is to determine calcium binding affinity for the binding pocket at the interface between extracellular domains 1 and 2. We monitored calcium titrations with circular dichroism and fluorescence spectroscopy. Binding affinities were different when spectroscopic techniques are compared to each another and a mutation to one tryptophan showed to be significantly debilitating to calcium binding.

LIST OF ABBREVIATIONS

NCAD	neural cadherin
ECAD	epithelial cadherin
NCAD12	neural cadherin, domain 1 + domain 2
ECAD12	epithelial cadherin, domain 1 + domain 2
MECAD12	ECAD12 + methionine group
EC	extracellular
W2	tryptophan, second residue in NCAD sequence
W113	tryptophan, 113th residue in NCAD sequence
W2A	protein in which W2 is replaced with the amino acid alanine
W113A	protein in which W113 is replaced with the amino acid alanine
F	phenylalanine
W113F	protein in which W113 is replaced with the amino acid phenylalanine
CD	circular dichroism
NMR	nuclear magnetic resonance
SEC	size exclusion chromatography
APO	state of protein with no calcium added
SATURATED	state of protein when calcium is bound to all binding sites

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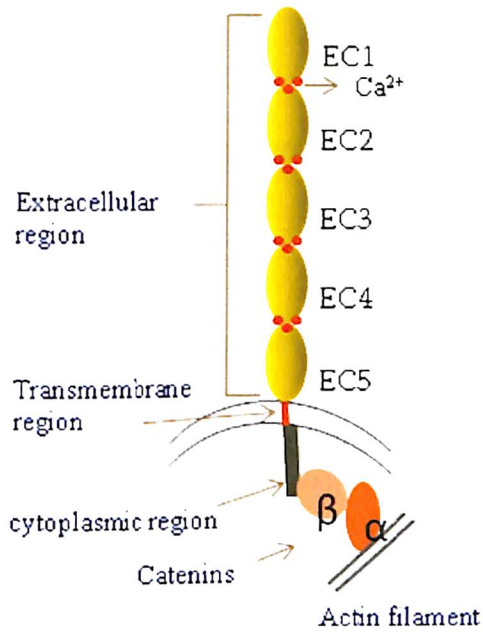
I. Introduction

A.) General Concepts of Cadherins

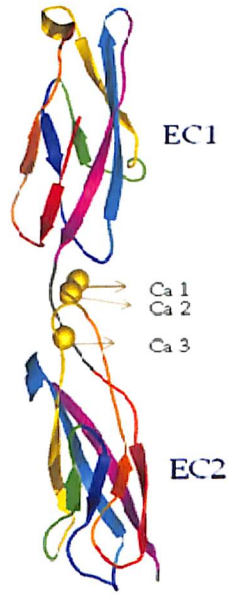
Cadherins are glycoproteins that extend through the cellular membrane on both sides and mediate cell-cell adhesion through homophilic, between identical molecules, connections [1-5]. Classical cadherins, Type I, are named depending on the cell type in which they are found. Neural (N-), Epithelial (E-), Retinal (R-), and Placental (P-) cadherins comprise the most well studied members of the classical cadherin family. All cell types that form solid tissues express members of the cadherin family. Homophilic assembly means that cadherins define adhesion specificities for the majority of cell types. When these well characterized members are compared up to eighty percent of the amino acids are conserved and the intracellular domain shows the highest degree of conservation [3].

Classical cadherins have analogous domain organization and conserve key amino acids that affect calcium binding [6-8]. Five extracellular domains, a transmembrane domain, and a C-terminal intracellular region compose the primary structure of cadherins. The C-terminal intracellular region interacts with the actin cytoskeleton by way of catenins [9] (Figure 1A). The ectodomains (EC in Figure 1A) of the extracellular

region are made of five tandemly repeated domains of approximately 110 amino acids consisting of seven-stranded antiparallel β- barrels organized into two opposing sheets (1, 2) (Figure 1B). The domains are similar in amino acid sequences and structure. The extracellular domains are numbered 1 through 5 with EC5 as the membrane proximal domain and EC1 as the N-terminal domain. The ectodomains form lateral dimers between cadherins located on the same cell surface. Adhesive dimers are formed using the ectodomains between identical cadherins located on neighboring cell surfaces and results in cell-cell adhesion (Figure 2) [12, 13].



(A)



(B)

Figure 1: (A) Basic cadherin structure showing extracellular ectodomains (EC), transmembrane region, and intracellular cytoplasmic region. Orange dots represent calcium bound to linker regions between the 5 ectodomains. (B) Ribbon drawing of EC1, EC2, and the calcium binding linker between them in Epithelial-cadherin as produced by *Nagar et al 1996* [45].

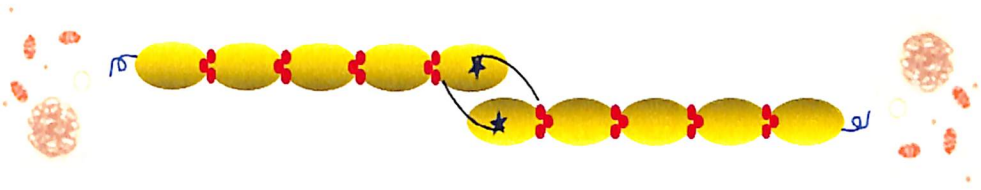


Figure 2: Adhesive dimer formation between neighboring cadherin cells

Cadherins are used widely throughout the body for a variety of different physiological processes. Cadherin expression is developmentally regulated and the turning on and off of cadherin expression correlates with cell aggregation or disaggregation in many morphogenetic events [14]. Cadherin expression is significantly higher in closely aggregating cells compared to nonaggregating cells, thereby showing a direct relationship between cadherins and cell adhesion [3]. Cadherins are very important in embryogenesis [15, 16], cell sorting [17, 18], and cell differentiation [19]. Cadherin proteins also have fundamental roles in neural development, synaptogenesis, synapse maintenance [20], synaptic plasticity [21, 22], and tumor progression and metastasis [23]. Cadherins are dependent on calcium for cell-cell adhesion. They are considered the most important for the formations of physical cell-cell associations, because as long as cadherins are functioning, inactivation of other adhesion systems has minimal effect on cell-cell adhesion [24, 25].

B.) N-CAD Specific

Neural-cadherin (N-CAD) is expressed in the central nervous system along with epithelial cadherins, but is segregated to specific regions of the brain, neuron, and

different synapse types [26, 27]. Studies have shown that domain 1 of NCAD regulates the specificity of homophilic interactions. Exchanging domain 1 of epithelial- and neural- cadherins switches their specificity [28], and antibodies to domain 1 inhibit adhesion completely [29, 30]. Specific deletion [28] or mutation [31] of domain 1 of N-CAD negates cell-cell adhesion.

In addition to binding specificities, NCAD plays different roles within the nervous system. NCAD expression is known to initiate during the formation of the neural tube and peripheral ganglia [32-36]. N-CAD transsynaptically regulates short-term plasticity at glutamatergic synapses in embryonic stem cell-derived neurons [21]. N-CAD also plays an important role in conducting electrical impulses over long distances in the central nervous system [22, 37]. N-CAD is found at dendritic spines of neurons where the excitatory synapses are located and at dendritic shafts, soma and near-by axonal regions where inhibitory synapses of neurons are located [38-42]. Previous literature has implicated that possibly the adhesive specificities of N-CAD could regulate certain aspects of central synaptic junction formation [38].

Studies have shown that introduction of an N-CAD antibody can destroy the structure of the retina almost entirely in early stages, but only partially at later stages of development [43]. This shows that N-CAD proteins have importance during development of different tissue types and that N-CAD might differentiate into different cadherin types not yet well characterized, such as retinal-cadherin. N-CAD is expressed in melanocytes and progenitor cells in the human limbal epithelial stem cell niche and

may be a critical cell-cell adhesion molecule for repairing damaged corneal surfaces [44].

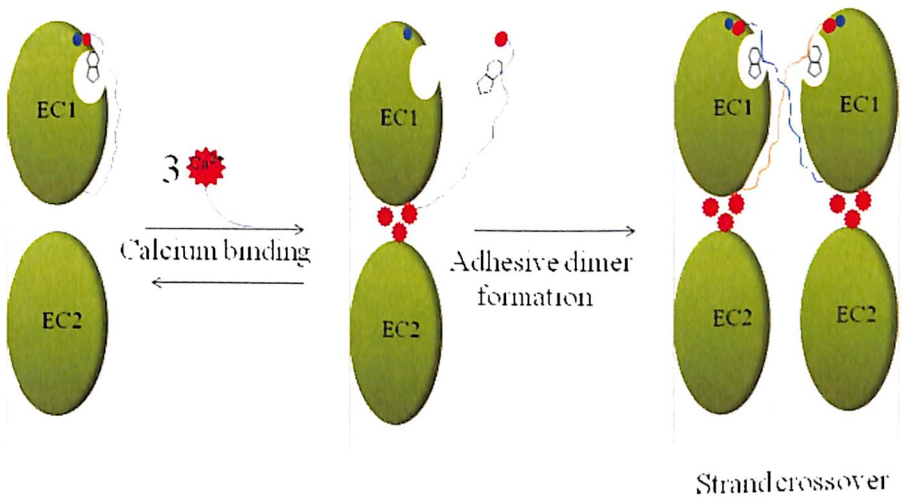
C.) Model of W2 exposure

Isolated extracellular domains of N-CAD are known to fold independently and early high resolution studies provided agreement on certain characteristics. Characteristics supported by NMR spectroscopy and X-ray crystallography include: 1.) Extracellular domains of N-CAD are composed of seven β-barrel strand (strands A through G) structures. 2.) The A-strand is most proximal to the N-terminus and is irregular due to a proline-proline (Pro5-Pro6) connection that disrupts the hydrogen bonding to strand-G and/or strand-B. 3.) Identical interface regions of the domains are where calcium binds [31, 45, and 46]. 4.) There is a tryptophan residue, W2, in the second position of the primary sequence of the monomer that is the correct size to occupy a hydrophobic pocket in domain 1.

The crystal structure of N-CAD domain 1 showed a *cis*-dimer interface between two subunits where the W2 of one monomeric subunit was inserted into the domain 1 hydrophobic pocket of the neighboring subunit [47]. This crystal structure showed strand-A hydrogen bonding with strand-B of its neighbor and then with its own strand-G after the Pro5-Pro6, and forms what has been named a "strand dimer." There was no strand-dimer formation found in domain 2 [31].

Supporting this strand-dimer are more recent NMR and crystallographic studies that show a strand-crossover dimer by means of the βA-strand detaching from domain 1

and exposing a tryptophan, W2. The βA-strand detaching allows the W2 from one subunit to insert or dock into the hydrophobic pocket of a neighboring subunit and form a strand-crossover (Figure 3). The adhesive dimer interface is thought to be this strand-crossover complex [48. 49]. Calcium has been shown to change interactions with antibodies that change responses to the environment surrounding the W2 and change the structure of the hydrophobic pocket [50]. Troyanovsky *et al* showed W2 was required for lateral and adhesive dimer formation between cadherins prepared from mixed cell cultures [51]. Adhesive dimer formation has been shown to require interactions between the side chain E89 and the N-terminus of domain 1 [12, 13].



Source: Harrison, O.J., et al., *The mechanism of cell adhesion by classical cadherins: the role of domain 1.* J Cell Sci, 2005. **118**(Pt 4): p. 711-21.

Figure 3: Testable model for strand-crossover dimer formation. Calcium binding to the interface between domain1 and domain2 results in detachment of the βA-strand and exposure of the N-terminus and W2 ( ). The strand-crossover dimer is created by the βA-strand forming noncovalent, interdomain interactions with the neighboring cadherin. The red circle represents the N-terminus, blue circle represents side chain E89, and red sun bursts represent Calcium ions. The actual orientation of the monomers is unknown.

These studies from the literature provided a testable model for the mechanism of strand-crossover dimer formation. This model suggests that the Apo, without

calcium, or "closed" state is made possible by the intradomain interactions of W2 docking into its own hydrophobic pocket and ionic stabilization between the N-terminus and side chain E89. Calcium binding detaches the βA-strand which reveals the N-terminus and W2. During this "open" state two cadherins would form the strand-crossover dimer when the W2 is docked into the neighboring hydrophobic pocket and ionic interactions are formed, resulting in interdomain interactions. The orientation of the two cells forming the strand crossover was not analyzed. Chen *et al* propose the possibility that calcium binding is necessary to surmount the energetic barrier of exposing the βA-strand [52]. A testable model for strand-crossover dimer formation is shown (Figure 3).

D.) Binding

Binding reactions are used to describe and measure the functional chemistry of macromolecules and are represented pictorially through binding curves. Binding curves are used to express quantitatively the binding process while taking into account the amount of ligand bound and the activities of various ligands. For the purpose of these experiments calcium was the ligand binding to the cadherin macromolecule and was analyzed as binding occurred to determine the average affinity of the cadherin.

The average binding affinity was determined by using the fractional saturation at the midpoint of the binding curve,θ, of a protein for calcium with the simple equation below

$$\theta = \frac{Kx}{1 + Kx} \qquad \textbf{Equation 1}$$

where K is the equilibrium constant formed between calcium ligands free and bound and x is the concentration of the calcium ligands in Equation 1. Binding curves for these titration experiments are plotted as signal versus the logarithm of the calcium concentration of the solutions. The shape of the titration curves illustrates the macromolecular binding process data plotted versus a logarithmic scale [53]. Three calcium ions are known to bind to the interface between extracellular domains in classical cadherins, and the order in which the ions bind might affect exposure of the W2, but is beyond the scope of this work.

E.) Spectroscopy

Calcium titrations presented in this work were measured using circular dichroism and fluorescence spectroscopy. Circular Dichroism (CD) is measured by passing left circularly polarized light and then right circularly polarized light through a sample and obtaining the difference between the signals [54]. Cadherins are proteins that have many different types and numbers of amino acids. Amino acids have a chiral carbon and because of this their secondary structures are chiral and absorb polarized light. CD acts as a relatively sensitive probe to cadherin conformation. Cadherins' secondary structure is mostly β-sheet and yields a much lower CD signal (at approximately 218 nm minimum) than α-helices do. Negatively-charged residues or carbonyl groups on the loops and linker regions of N-CAD12 bind calcium and change the disposition of the

secondary structure which in turn changes the conformation of the entire protein. This conformational change will be manifested as changes in the CD signal.

Fluorescence spectroscopy works on the principle of Planck's law. Energy absorbed by a molecule will be emitted in relation to specific quantized transitions between energy levels [54]. N-CAD12 has two tryptophan residues, W2 and W113, and five tyrosine residues. All seven of these residues will absorb UV light at approximately 280 nm, due to conjugated double bond systems within their amino acid structure, while only the two tryptophans will absorb UV light at 290 nm. When the UV light is emitted, or fluoresced, it will do so in a particular wavelength range. By analyzing the maximum wavelength of fluoresced UV light after exciting the molecule at 280 nm and 290 nm, the exposure and/or docking of W2 might be seen as calcium is titrated into the solution as a shift to a higher max wavelength.

CD spectroscopy will show global changes in the secondary structure of the protein that might be present without a changing environment surrounding the tryptophan. Fluorescence spectroscopy will monitor local change in the environment surrounding tryptophan by looking at the wavelength where the tryptophan fluoresces.

F.) Experiment Description

In order to analyze the calcium binding properties of N-CAD12 the gene was first placed in an engineered vector. Two mutant forms were developed in addition to the wild-type. The two mutant forms of N-CAD were made by point mutating the tryptophan residues in the 2 and 113 positions of the cadherin amino acid sequence to

alanine. The three forms of NCAD12 tested were the wild-type (wt), w2a, and w113a. In the NCAD12-w2a mutant the tryptophan residue in the second position is swapped for alanine so that only the tryptophan in the 113 position will be studied. The same approach applies for NCAD12-w113a so that the tryptophan in the 2 position can be solely studied. Calcium titrations of the three N-CAD12 forms were observed spectroscopically. These studies assess if calcium binding causes W2 exposure and provides estimates of the calcium binding affinity for each protein.

G.) Importance of Studies

The model of strand-crossover dimer formation has never been rigorously tested in N-CAD12. In literature, "docking" of W2 has not been demonstrated under "normal" buffer conditions for either N- or E-CAD. The function calcium plays in conformation changes of cadherins has not been shown. Since the isolated extracellular domains of cadherins fold independently their properties in solution will serve as a simplified system for cadherin folding, structure and assembly *in vivo* [55].

In epithelial-cadherin the homophilic and heterophilic, between different molecules, interactions depend on proper binding of calcium to the interface between domain 1 and domain 2 [55]. This research will allow for the first ever analysis of W2 exposure of N-CAD12 through calcium titrations monitored for fluorescence of the tryptophan residues. Also the conformational changes that calcium induces in N-CAD will be shown and analyzed through calcium titrations monitored by circular dichroism. The results from this research will allow comparisons between newly characterized properties of N-CAD calcium binding monitored under CD and fluorescence spectroscopy. Differences in calcium binding between CD and fluorescence techniques will allow for a new model of W2 exposure to be created for further testing.

II. Materials and Methods

A.) Protein Preparation

The original N-CAD clone was kindly provided by Dr. Shapiro's lab. The gene for N-CAD12 was subcloned into a pET30b vector and contains the region for domain 1, linker 1, domain 2, and linker 2. Amino acid residues 1 through 219 are shown (Figure 4). N-CAD12 was overexpressed using the BL21 (DE3) (*E. coli*) expression cell line. The point mutations to substitute alanine for tryptophan residues, NCAD12-w2a and NCAD12-w113a, were completed by graduate student Nagamani Vunnam using a Quick-Change Mutagenesis Kit by Stratagene. The overexpression and purification process is described in previous literature [56] with these certain alterations following. Amicron Ultra 10,000 MWCO Millipore centrifugal concentrators were used to concentrate the elution fractions that possessed N-CAD12. Size exclusion chromatography (SEC) was used to transfer the concentrated N-CAD12 into the working buffer by using a Sephacryl S-100 column with a flow rate of approximately 0.5 mL/min.

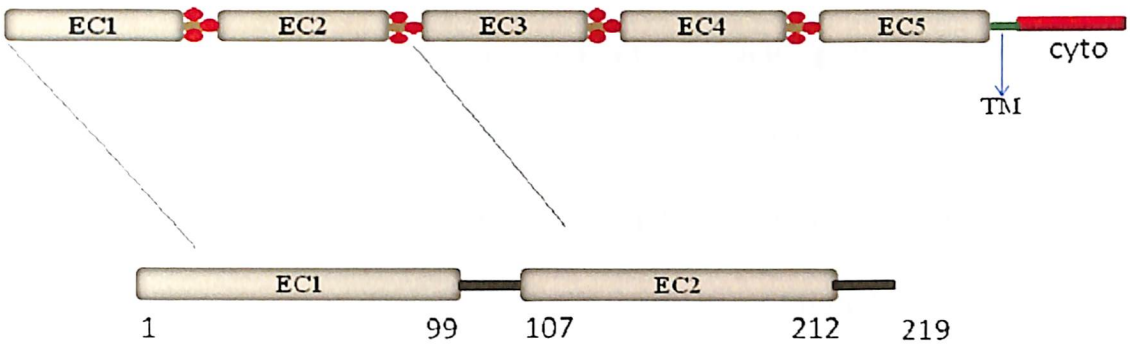


Figure 4: *Extracellular domains of N-CAD as cloned for experimentation. Calcium is represented as red dots bound at the linker regions between domains. Studied in this research are domains 1 and 2 of Neural-cadherin with the adjoining linker segments (NCAD12). This represents the smallest functional unit to study cell-cell adhesion and calcium binding.*

Spectral studies were performed with protein in buffer solution of 10 mM HEPES, 140 mM NaCl, pH 7.4 (SEC Buffer). Protein solutions were made by diluting NCAD12-wt, NCAD12-w2a, and NCAD12-w113a stocks with SEC buffer. For titrations, 1 mM and 10 mM calcium chloride stocks were produced from 100 mM calcium solution (Thermo Electron Corporation). High concentration calcium solutions were 0.7 M $CaCl_2$.

B.) Circular Dichroism

Calcium titrations were performed by creating protein solutions of 1.5 mL with 5 μM protein in SEC buffer. The solution was placed in a 0.4x1cm quartz cuvette and read using an AVIV 202SF circular dichroism spectrometer with CDS 3.02A software made by AVIV Biomedical, Inc. Specific experimental variables for the AVIV 202SF were replicated for each titration. The pathlength of the cuvette was 1 cm and the stir control was on and had a reading delay of 100 milliseconds. The experiments performed were wavelength step scans that started at 300 nanometers (nm) and ended

at 220 nm with a data point taken every 1 nm with an averaging time of 5 seconds. The temperature was held at 25°C and the time constant was 100 milliseconds. The same cuvette was used for all CD-titrations and was washed twice with tap water then twice with SEC buffer prior to each experiment. The protein was titrated with small volumes (in order 2 µL, 5 µL, and 10 µL) of four different calcium concentrations (1 mM, 10 mM, 100 mM, and 0.7 M). The three increasing volumes were administered by Finnpipettes (Labsystems) for each calcium concentration, in increasing order. For example, 2 µL, 5 µL, and 10 µL of 1 mM calcium were the first three additions. These volumes of additions were used in this order to ensure evenly spaced data on a logarithmic scale. Scans with only SEC buffer and with no calcium added were made prior to calcium additions. The same two Finnpipettes, 0.5 to 10 µL and a 20 to 200 µL, were used to add calcium stocks and mix the solution in the cuvette after calcium injection. The total calcium concentration was assumed to be equivalent to the free calcium concentration since the experiments were run at low protein concentration. Approximately 1 minute was allotted after each calcium addition before beginning the CD scan. The spectral data were corrected for differences by subtracting the signal of the SEC buffer scan from the titrated protein signal, and then offset corrected by subtracting from each blank corrected data point the average of the blank corrected signal from 296 to 300 nm. The calculation for the corrected signal is given by the following equation.

$$Corrected\ signal = \textbf{Sample signal} - \textbf{Blank signal} - \textbf{Offset signal} \quad \textbf{Equation 2}$$

C.) Fluorescence

Intrinsic tryptophan fluorescence was assessed using a PTI QuataMaster™ spectrofluorometer system outfitted with an A1010B arc lamp and Felix 3.2 version 1.00 software. Emission scans were acquired from 300 to 400 nm and excitation was set at 280 nm and 290 nm for each calcium addition. The scan step size was 1 nm, the integration time and averaging time was 1 second. Scans were made at 25°C and evaluated at the excitation and emission polarizer angle of 54.7°. Two milliliters of 2.78 μM protein solution was prepared with SEC buffer for each titration of each protein type and placed into a quartz cuvette for titration. The protein solution was titrated with calcium by small volume additions of calcium chloride as described in the CD method. Approximately two minutes was allowed to elapse between when calcium was injected and the wavelength scan began. The raw data obtained from the spectrofluorometer was "smoothed" in Microsoft Office Excel. Data was smoothed by averaging the raw data from five wavelengths and the average would become the smoothed data value for the third wavelength of the five points that were averaged. For example, the smoothed data value at 302 nm is the average of the raw data values from 300 to 304nm. The centroid, central wavelength of the titration, was calculated by the equation below and allowed for a reference point besides the max wavelength.

$$Centroid = \frac{\Sigma(\text{Signal} \cdot \text{Wavelength})}{\Sigma(\text{Signal})}$$

Equation 3

The maximum wavelength was found by locating the highest fluorescence signal in the smoothed data.

D.) Titration curves

CD and fluorescence signals were plotted versus the log of the calcium concentration to obtain titration curves. Microsoft Excel was used to combine data from all wavelengths scanned during the titrations and to blank and offset correct that data. Microsoft Excel was also used to plot initial titration curves to see which wavelengths should be considered for more advanced analysis. Igor Pro version 4.01 (Wavemetrics) was used to analyze the titration curves. Each titration curve was analyzed for having a noncooperative model (Equation 1) yielding free energies, slope and intercept values at apo and saturated baselines, and chi-squared values. Each titration curve was analyzed by Igor Pro with the slopes fixed to 0 and the intercepts were allowed to vary. The titration curve for NCAD12-wt monitored under fluorescence was analyzed both with the slopes fixed to 0 and allowed to vary.

III. Results of Spectroscopic Studies

A.) Titrations Monitored by CD

1.) General Trends

Representative calcium titrations of NCAD12-wild type (wt), NCAD12-w2a, and NCAD12-w113a are shown in figures 5 through 7. The CD signal for each protein type increased (became less negative) as calcium was added at each of the wavelengths monitored. However, the CD signals differed in value between the different forms of NCAD12. At 229 nm, the wild-type CD signals started at near zero values and ended at positive values (Figure 5). At 229 nm, The NCAD12-w2a CD signals started at negative values and ended near the start values of the NCAD12-wt (Figure 6), and the NCAD12-w113a started at the lowest (most negative) values and ended proximal to the NCAD12-w2a start values (Figure 7). This trend of wild-type, w2a, and w113a decreasing in CD signal strength, compared to each other respectively, held true at each wavelength. Also, for each protein type the CD signal increased as calcium concentration increased through titration. The best titration data consistently appeared between a wavelength range of 232 nm to 225 nm while above and below this range signal noise was significantly increased.

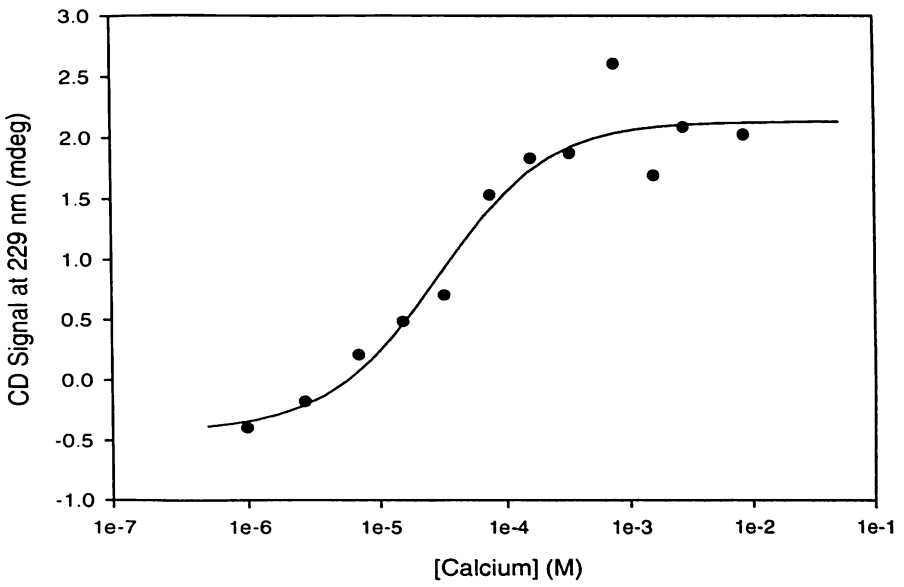


Figure 5: CD titration of NCAD12-wt from the first experiment. This data is observed at 229 nm and yielded a free energy change of -6.2 ± 0.2 kcal/mol.

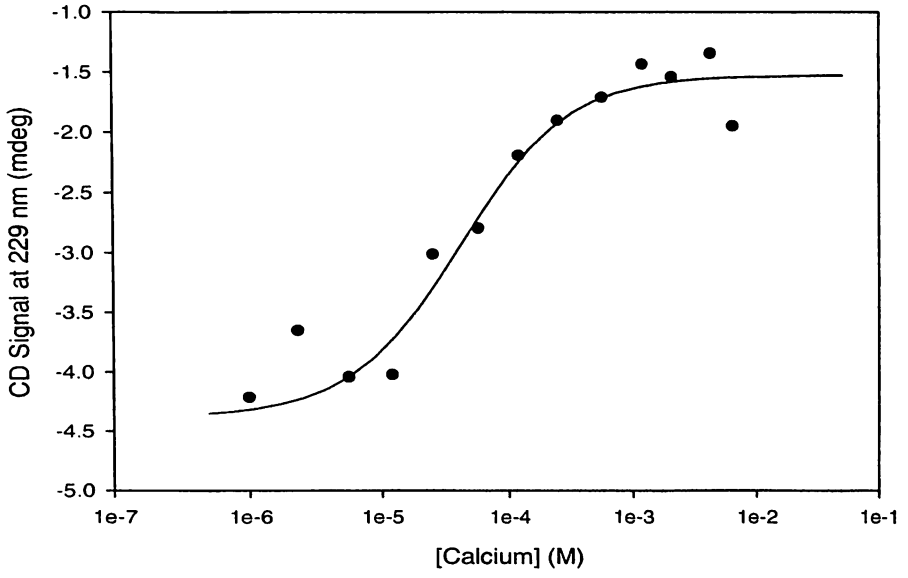


Figure 6: CD titration of NCAD12-w2a from the first experiment. This data is observed at 229 nm and yielded a free energy change of -5.9 ± 0.2 kcal/mol.

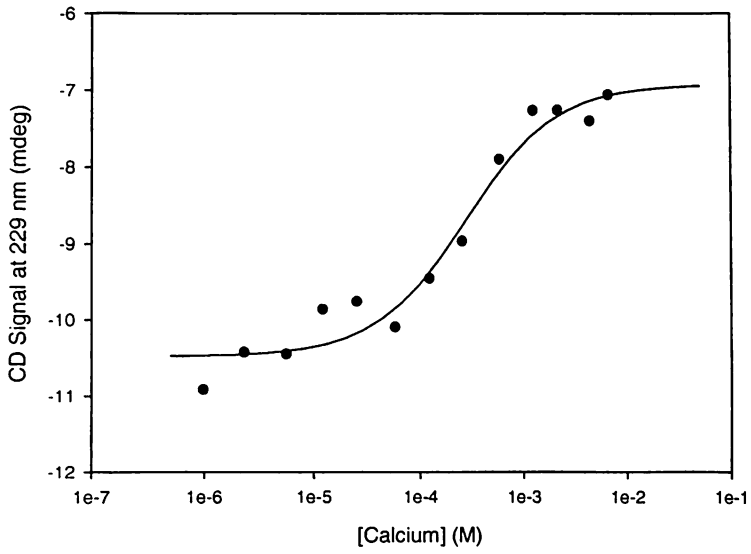


Figure 7: CD titration of NCAD12-w113a from the first experiment. This data is observed at 229 nm and yielded a free energy change of -4.9 ± 0.2 kcal/mol.

2.) Comparisons of Results

Best fitted values for free energy change for the protein types at 229 nm are located in **Table 1** and were obtained by fitting the CD data to a simple model in which the sites are equal and independent of each other (Equation 1). Typical data are shown for each protein. NCAD12-wt and NCAD12-w2a have similar affinities with best fit values of free energy change differing by only 0.2 kcal/mol. This is a small difference given the uncertainty in the resolved free energies (0.2 kcal/mol). In contrast, NCAD12-w113a has significantly lower binding affinity with approximately 1 kcal/mol less energy released upon calcium binding. A statistical hypothesis test, T-test, was applied to the free energy values of NCAD12-wt and NCAD12-w113a, which make 2 degrees of freedom, to determine if the difference was statistically significant.

The equation for the T-test is

$$T = \frac{Y1avg - Y2avg}{\sqrt{(S.D.1)^2 + (S.D.2)^2}} \qquad \textbf{Equation 4}$$

where Y1 is the average CD free energy of NCAD12-wt, Y2 is the average CD free energy of NCAD12-w113a, S.D.1 is the average standard deviation of Y1, and S.D.2 is the average standard deviation of Y2. The T value is compared to a T-chart which takes into account degrees of freedom and the percent confidence in the statistical difference can be found. The T value was found to be 4.60 and for 2 degrees of freedom gives us greater than 95% confidence that the difference between the free energy values of NCAD12-wt and NCAD12-w113a is statistically significant [57].

Table 1: Free Energy Change of All Protein Types at Midpoint of Titration Curves in Replicated CD and Fluorescence Experiments

Protein	CD (Kcal/mol)	FL (Kcal/mol)
NCAD12-wt	-6.2 ± 0.2	-5.4 ± 0.3
NCAD12-w2a	-6.0 ± 0.1	-5.7 ± 0.1

NCAD12-w113a	-4.9 ± 0.2	N/A

B.) Fluorescence

1.) General Trends

Significant change is noticed through the calcium titrations of the various NCAD12 forms monitored by fluorescence spectroscopy. Calcium titrations were analyzed at excitation wavelengths of 280 nm and 290 nm. The maximum emission for each excitation wavelength in Apo solutions was selected. Emission data for each subsequent calcium addition was plotted versus the log of the calcium concentration to form titration curves. The maximum wavelength remained consistent at both 280 nm and 290 nm excitation for each protein type, but differed between the types. The maximum wavelength for NCAD12-wt was 330nm. The maximum wavelength for NCAD12-w2a was red-shifted, compared to NCAD12-wt, to 335nm. The maximum wavelength for NCAD12-w113a was blue-shifted, compared to NCAD12-wt, to 320nm (Figure 8). The maximum wavelength of NCAD12-wt is closer in value to the protein with the tryptophan only in the 113 position.

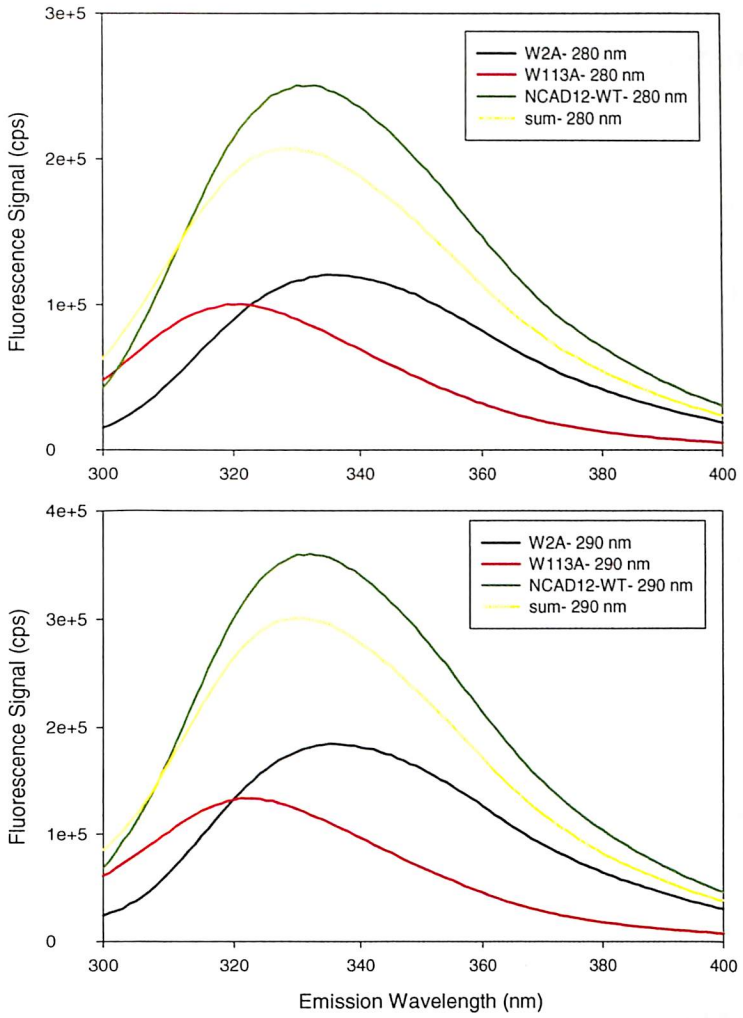


Figure 8: Fluorescence spectra of all NCAD12 protein types at both 280 nm and 290 nm excitations.

2.) Comparison of Results

When the maximum wavelength is plotted against the log of the calcium concentration the curves produced for NCAD12-w113a are relatively linear and decrease in fluorescence signal as calcium concentration increases at both 280 nm and 290 nm excitation (Figure 9). Due to both the 280 nm and 290 nm excitation fluorescence signals of NCAD12-w113a decreasing in a very similar manner, only the

280nm excitation graph is shown. Since the NCAD12-w113a curves are linear they cannot be fitted by Igor Pro for curve analysis and free energy values are not obtained.

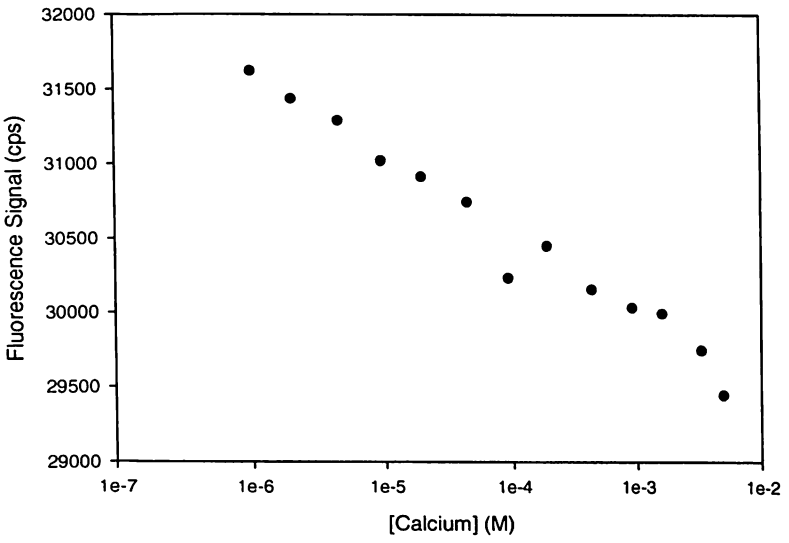


Figure 9: Fluorescence titration of NCAD12-w113a at 280 nm excitation. These data are observed at 319 nm. This same trend was observed at 290 nm excitation except at a fluorescence signal range of 44000 to 40500. These data were not fit to Equation 1.

In contrast, the titration curves for NCAD12-w2a look relatively sigmoidal at 280 nm and 290 nm excitation and can be curve-fitted by Igor Pro (Figure 10). Free energy values for NCAD12-w2a are presented in Table 1. Notice that the best fit value is 0.3 kcal/mol less for the fluorescence data than for the CD data.

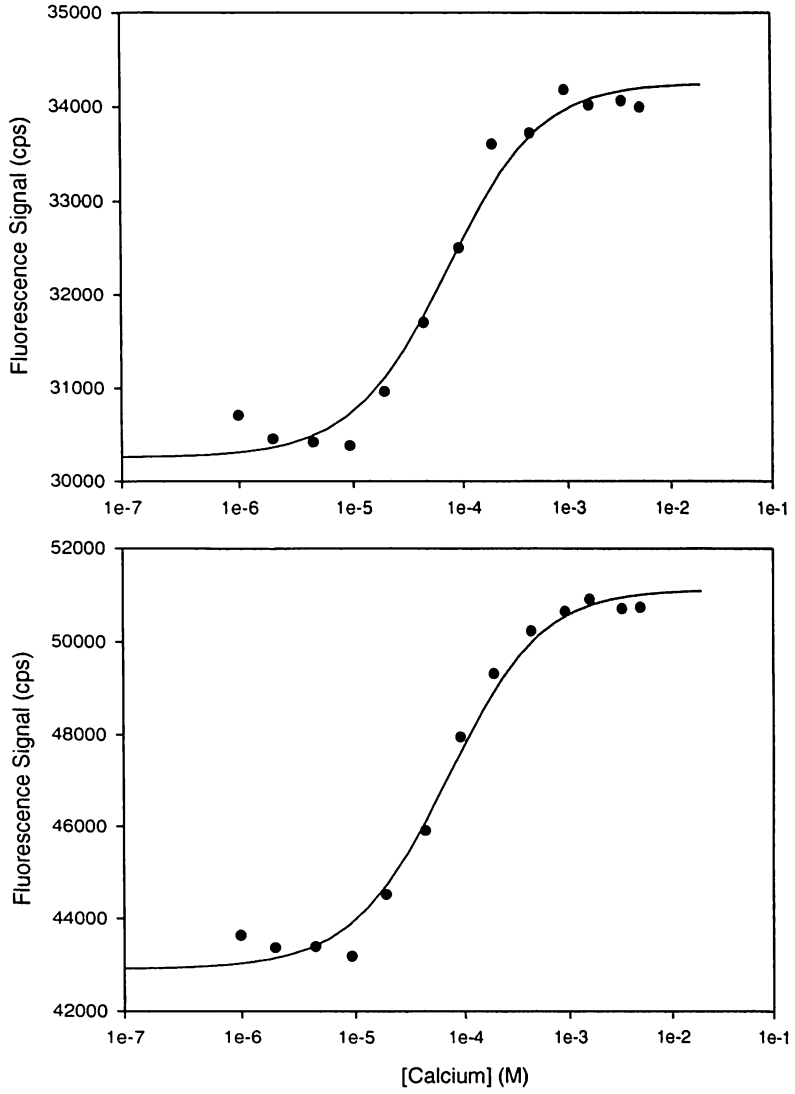


Figure 10:

Top- Fluorescence titration of NCAD12-w2a at 280 nm excitation. This data is observed at 335 nm and yielded a free energy change of -5.7 ± 0.1 kcal/mol.

Bottom- Fluorescence titration of NCAD12-w2a at 290 nm excitation. This data is observed at 335 nm and yielded a free energy change of -5.7 ± 0.1 kcal/mol.

The two calcium titrations of NCAD12-wt monitored for fluorescence produced data that are roughly the sum of the data for NCAD12-w2a and NCAD12-w113a. Data of

NCAD12-wt at 280 nm excitation showed a strong negative slope in both the presumed apo and saturated baselines. The data of NCAD12-wt at 290 nm excitation showed some titration by the shape of the titration curve from the first experiment, but showed no titration in the second experiment's titration curve (Figure 11). Only the data from the maximum wavelength of 330 nm for NCAD12-wt at 290 nm excitation from the first experiment was able to be curve-fitted by Igor Pro and revealed a free energy value of -5.4 ± 0.7 kcal/mol. The fit is poor, but the midpoint is the midpoint of the data. If baseline slopes are allowed to vary, the same value of free energy is returned, but the standard deviation is reduced to ±0.3 (Shown at bottom of figure 11). Due to the limitation of the data, confidence cannot be had in the fit, but the trend is interesting.

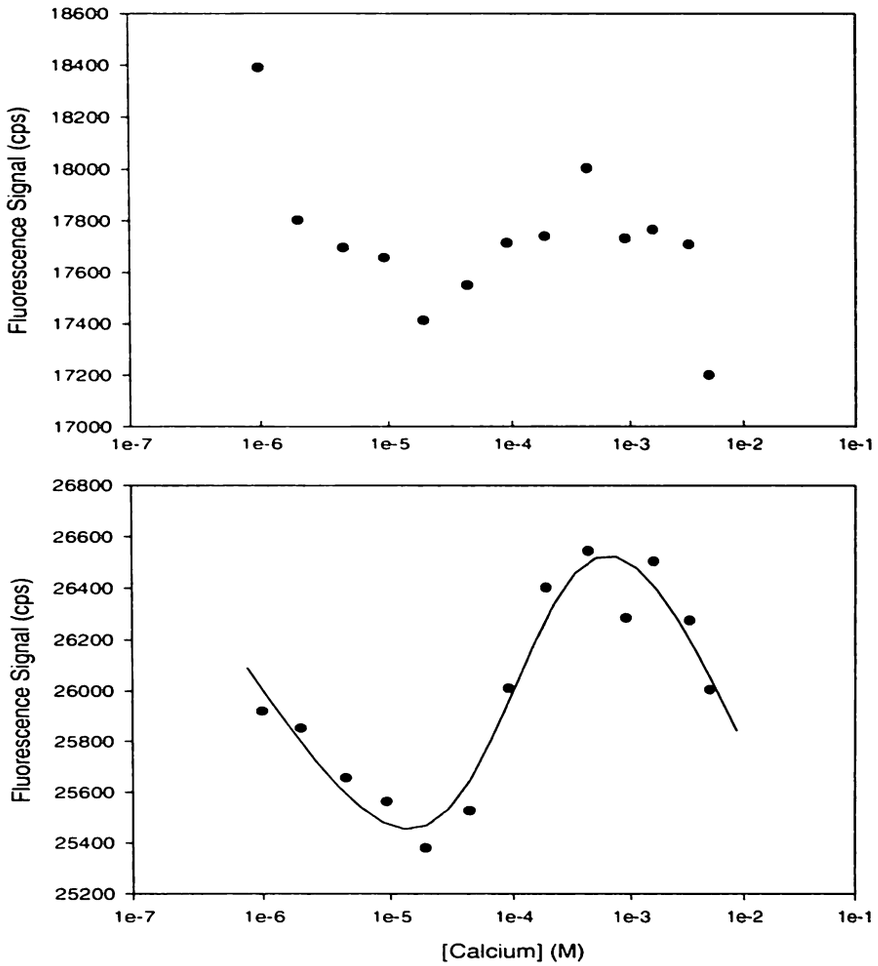


Figure 11:

Top- Fluorescence titration of NCAD12-wt at 280 nm excitation. This data was observed at 329 nm and a free energy change was not able to be calculated.

Bottom- Fluorescence titration of NCAD12-wt at 290 nm excitation. This data was observed at 330 nm and yielded a free energy change of -5.4 ± 0.3 kcal/mol.

IV. Conclusion

In these experiments, calcium binding affinity was measured by differences in free energy values obtained from analysis of calcium titrations observed under CD and fluorescence spectroscopic techniques (Table 1). The protein mutants differed in their free energy values when compared to each other and yielded several interesting insights.

Data from fluorescence titrations showed minimal change in wavelength, both centroid and maximum, as calcium concentration was increased in all protein forms. The maximum wavelengths remained consistent for individual protein types as calcium was added. Although the differences monitored were minimal for each protein form, they appeared at different wavelengths for the different protein forms. These data led us to conclude that W2 and W113 are in distinct environments with distinct fluorescence properties.

NCAD12-wt calcium titrations monitored by CD spectroscopy produced very similar binding constants to MECAD12, ECAD12 with a methionine group added. The signals for NCAD12-wt are produced from the W2 and W113 amino acids. NCAD12-wt monitored by fluorescence spectroscopy yielded a poor calcium dependent signal. In

addition, data for NCAD12-wt did not show obvious exposure of W2. Monitoring NCAD12-wt by fluorescence spectroscopy leads to lower free energy transitions when compared to that of the CD data (Table 1). This might imply that the local environments of the Tryptophan residues are sensitive to a lower affinity calcium binding event than the global conformational change as monitored by CD.

When monitoring the NCAD12-w2a protein mutant under spectroscopy the signal is produced from only the W113 residue. W113 undergoes a large change in environment upon binding of calcium when monitored by fluorescence. There was no maximum wavelength change, but a large fluorescence intensity change occurred. The titrations of NCAD12-w2a produced excellent reproducible titrations under fluorescence spectroscopy. The NCAD12-w2a mutant bound calcium with a lower affinity (0.2 kcal/mol) than NCAD12-wt did in best fit values. This shows that mutation has minimal affect on binding affinity. W2 exposure is linked to calcium binding so there should be an energetic linkage which might be reason for 0.2 kcal/mol difference from the NCAD12-wt. When best fit free energy values of CD and fluorescence are compared for NCAD12-w2a there is a small, 0.3 kcal/mol, difference with the fluorescence signal being lower (Table 1) even when the error is taken into account. CD monitors the global change of the secondary structure and fluorescence monitors local changes surrounding the W113 residue. Similar to NCAD12-wt the fluorescence data shows local changes at higher calcium concentrations than the major conformational changes in the construct.

The CD and fluorescence free energy data for NCAD12-wt were compared against each other using the T-test, equation 4, to determine if the differences were statistically significant. The same procedure was applied to CD and fluorescence free energy data of NCAD12-w2a. The T value for differences between CD and fluorescence free energy change of NCAD12-wt was 2.22 and was 2.12 for NCAD12-w2a. These T values assessed for 2 degrees of freedom give us less than 90% confidence that the difference between CD and fluorescence free energy change is statistically significant [57].

The signals produced by NCAD12-w113a are due to only the W2 residue. We expected a large change in wavelength max upon addition of calcium due to the W2 exposure [58]. No change in wavelength max was observed. This might be due to the W113 being eliminated and not allowing for the proper calcium to bind to expose the W2, which would cause the wavelength maximum to shift. The mutation to W113 lowered the calcium binding affinity by 1.3 kcal/mol as compared to NCAD12-wt. This large perturbation in binding energy was not expected and shows that this mutation is significantly debilitating. Fluorescence signals might not have been observed for NCAD12-w113a due to impaired calcium binding properties. Calcium was added to the point of saturation according to the CD signal, but this might not be a high enough concentration to bind the proper calcium to cause a fluorescence signal change.

In conclusion, the data yielded in increasing order of calcium binding affinity or decreasing order of free energy of the reaction w113a, w2a, and wild-type protein

forms, as seen in Table 1. This shows that the tryptophan in the 113 position affects calcium binding more than the tryptophan in the 2 position. Since W113 affected the binding of calcium significantly it might have a direct effect on the local environment of the W2. Since W2 exposure is linked to calcium binding, mutating W113 could be a reason why no exposure of W2 was seen. W2 exposure might not have been observed in NCAD12-wt due to the calcium concentration being insufficiently low to bind the calcium required for W2 exposure. Since differences between CD and fluorescence free energy changes were observed a new model of W2 exposure was created (Figure 12).

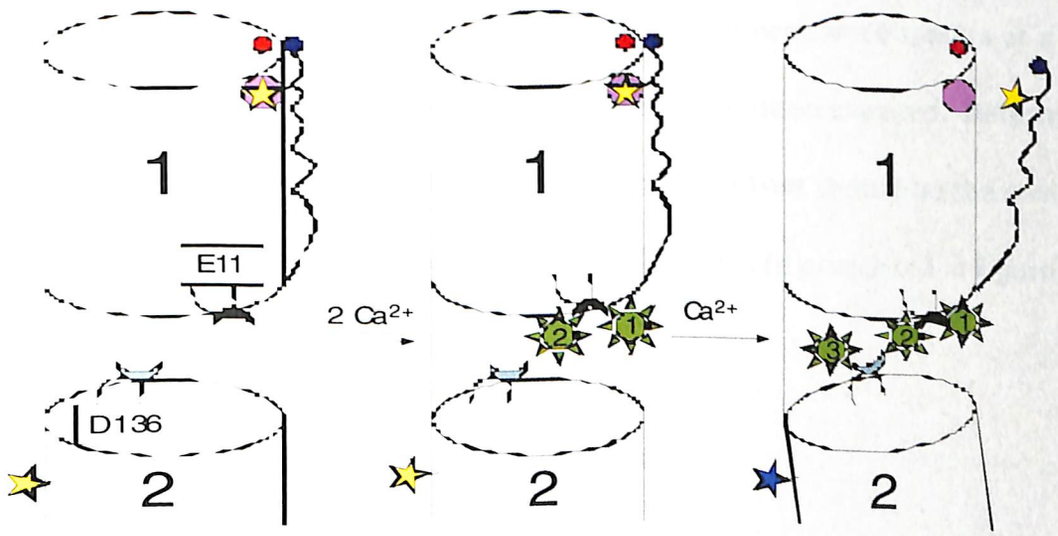


Figure 12: New model of W2 exposure

This new model takes into account two separate calcium binding events. The first binding event changes the global conformation of NCAD12 which is observed through CD signal change as calcium is added. The second binding event changes the

local environment of W113, which was shown to be primarily responsible for the fluorescence character of NCAD12-wt, and is observed as change in the fluorescence signal. The binding events together presumably cause exposure of W2, yellow star on Domain 1 Figure 12, although we have no experimental evidence of this. The figure might be misleading due to the fact that the stoichiometry of the number of calcium molecules binding during each event is currently under much debate.

Further studies are needed to provide conclusive data about W2 exposure. The data showed W113A mutation affected calcium binding properties. Phenylalanine, F, is structurally more similar to tryptophan, W, than alanine, A, and F is the residue in the 113 position in ECAD. The calcium affinity for NCAD12-w113f compared to NCAD12-wt should be similar under CD spectroscopic studies and fluorescence spectra as a function of calcium concentration should reveal whether W2 is indeed exposed. Determining the calcium affinity for NCAD12-w113f compared to NCAD12-wt should be the next step in research along with testing the new model of W2 exposure presented in Figure 12.

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