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Dimerization of Neural Cadherin in the Presence of Low Calcium

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by
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For my parents, family, and friends for their everlasting support.

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ABSTRACT

KYLER TUCKER PISCIOTTA: Dimerization of Neural Cadherin in the Presence of Low Calcium
(Under the direction of Dr. Susan Pedigo)

Cell-cell linkage is a necessity for proper tissue function and stability in many biological systems. One moiety that allows for the proper linking of cells to one another is a family of molecules known as cadherins. This family of proteins can be found within the membrane of many different types of cells and the extracellular portions of the cadherin proteins interact with those of other cells to link adjacent cells together via the formation of a dimer complex. The presence of calcium (Ca^{2+}) is of crucial importance for the formation of this cadherin dimer. In this study, we focused on the dimerization of neural (N-) cadherin proteins in the presence of low calcium concentrations. Consideration of low calcium concentration is of particular importance due to the existence of N-cadherins at neurological synapses. N-cadherin is the marker for long term potentiation within synapses, an environment with exceptionally low Ca^{2+} concentration compared to that of normal extracellular space (40μm vs 1mM). We hypothesized that this low calcium content would decrease the fraction of cadherins that existed in the dimer conformation. In order to test this theory, we determined the equilibrium constants of N-cadherin at decreasing Ca^{2+} concentrations (1000μM, 100μM, and 40μM) at physiological pH. This experiment required using a chelator (EDTA) to trap the dimer for all three Ca^{2+} concentrations. Then, using size exclusion chromatography (SEC), the concentration of N-cadherin in the dimeric state was determined, from which the equilibrium constants were calculated. Circular Dichroism (CD) spectroscopy was also used to monitor the secondary structural changes of the N-cadherin protein at each Ca^{2+} concentration. Results from this experiment are consistent with a change in the equilibrium to favor the monomeric state of N-cadherin at the lower Ca^{2+} concentrations found in neurological synapses. This observation implies that

excitatory synapses have plasticity due to the weak dimerization affinity in the prevailing calcium concentration.

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Introduction

General Introduction to Cadherins

Within the body there exists a family of molecules known as cadherins which are transmembrane adhesion molecules. This family of proteins facilitates proper, functional cell to cell (cell-cell) adhesion as well as cellular signaling within tissues.[1] Calcium is required for cadherin-mediated cell adhesion in vivo.[2] Many different types of cadherins exist in the body, but all maintain a similar structure and function with only minor variances between them.[3] Although a range of cadherin molecules exists in biological systems, these structures have shown a tendency to interact preferentially with identical cadherins, thereby maintaining the ability to distinguish between other types of cadherins and cells.[4] Also, depending on the type of cadherin, the proteins have an essential role in the morphological development of organ systems during development as well as assist in homeostasis processes within fully developed tissues. As such, these transmembrane glycoproteins play a pivotal role physiology due to their primary role in cellular adhesion, signal transduction, and tumor cell suppression.[5] Our studies focus on a branch of the cadherin family called classical cadherins.

Classical cadherins are single-pass transmembrane proteins that are the critical component of Adherens Junctions. The N-terminus of cadherin is outside the cell, and the C-terminus is in the cytosol. The extracellular (EC) portion of the molecule has 5 repeats of a modular domain. The interface between these EC-domains is the site of calcium binding (**Figure 1**). The intracellular portion of the protein is anchored to the cell's cytoskeleton via intermediary proteins within the cell known as catenins. The cytoplasmic domain of cadherins

forms complexes with beta-catenin or plakoglobin (gamma-catenin) directly. These two complexes will then bind to alpha-catenins to form cadherin-catenin complexes (CCC) within the cell (**Figure 1**). The alpha catenin in the CCC then interacts with the actin filaments of the cytoskeleton thus anchoring cadherins to the cellular cytoskeleton.[1] The cell-cell adhesive structure creates tension in the cytoskeletal networks between adherent cells, and controls cell physiology and differentiation.[6] The formation of this cellular anchor is accomplished via tyrosine phosphorylation of the catenin which bonds with the C-terminus of N-cadherin. This phosphorylation is controlled by levels of phosphotyrosine phosphatase and tyrosine kinase. These two enzymes target catenin molecules and thus regulate their disassembly.[7] Importantly, if Adherens Junctions are not under tension, the beta-catenin is not sequestered in the adhesive complex. It can then translocate to the nucleus and function directly or as part of a complex that then regulates transcription of genes that allows remodeling of tissues. [8]

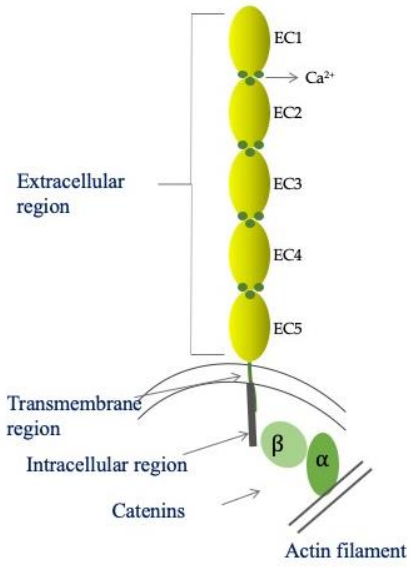

Figure 1: Schematic of catenin-mediated interaction between cadherin and the actin cytoskeleton.

Classical cadherins are a single polypeptide in which the N-terminus is outside the cell and the C-terminus is in the cytosol. They have 5 modular extracellular (EC) domains that bind calcium at the intersection between each of the domains. There is a single pass transmembrane helix. The cytoplasmic domain is linked to the actin cytoskeleton indirectly through beta-catenin and alpha catenin. This series of interactions ensures communication between adherent cells.

The ability to develop a model for cadherin-mediated cell-cell adhesion in a biological system has depended over the years on results from studies that employed a range of experimental methods including X-ray crystallography, chromatography, spectroscopy to study the protein-protein interactions that comprise the adhesive interface. One of the first proposed models, the cell-adhesion zipper, arose from an X-ray crystallographic structure that indicated the 3D structural details of the adhesive interface.[9] The structural data led to a proposed mechanism of adhesion between two cells that involved only direct contact between the N-terminal portion of the molecule. This dimerization interaction model was supported by a series of structural studies culminating in another, more complete, X-ray crystallographic structure of the entire extracellular domain of a representative of the classical cadherin family.[10] Details of these structural aspects are discussed in the next section. The beauty of structural studies is that they provide a model to test hypotheses regarding the specific relationship between structure and function.

Our work focusses on a particular member of the classical cadherin family, neural cadherin (N-cadherin), particularly in the role of calcium binding in its physiological function. Neural cadherin (N-cadherin) is present in the excitatory synapses between the neurons within the nervous system of the body.[11] During development neural cadherin manifests at the earliest stages of tissue development as the shape of tissues with complex morphology is being established.[12] It is present during angiogenesis as new blood vessels are infiltrating new or damaged tissues.[13] Our interest is its niche in the brain in synapses that are essential for long term potentiation,[14] the only place that we are aware of that has low levels of extracellular calcium.[15, 16] In this work, we study the effect of low levels of calcium on the equilibrium between neural cadherins *in vitro*.

Cadherin Structure and Function

The two most well studied members of the classical cadherin family are epithelial (E-) cadherin and N-cadherin. Their basic structure is outlined in the cartoon in **Figure 1**. The classical cadherin extracellular region contains the N-terminal end of the poly peptide consisting of five modular domains linked in series. Each of the EC domains contains approximately one hundred and ten amino acids. The sequence is conserved between different types of classical cadherin, and between EC domains in a particular cadherin, particularly for those amino acids that participate in the cadherin-specific adhesion events. It is important to note that the five extracellular cadherin repeat domains share the same folding topology termed a Greek Key motif (**Figure 2**). This protein fold is found in more than 700 proteins in the human genome.[17]

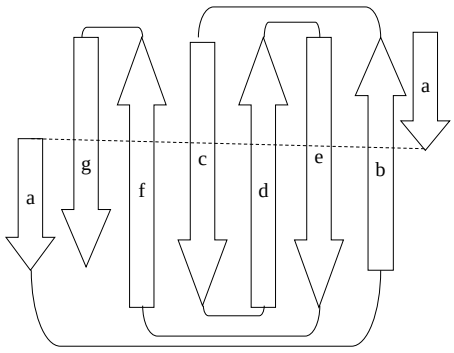

Figure 2: Topology of the modular domains of cadherins.

Each EC domain (EC1-EC5) has the same 7-strand topology, a so-called Greek Key motif. The folded domain is a beta-barrel, but flattened to such that the center of the domain is occupied by hydrophobic amino acids that form the hydrophobic core. The beta strands form antiparallel sheets except the C-terminal portion of the A-strand (parallel with G-strand, right).

A comparison of the amino acid sequences of E- and N-cadherins is shown in **Figure 3**. We focus our attention on only the first two EC domains because they are widely considered the minimal functional unit that forms dimer in a calcium dependent manner. The EC1 domain contains the adhesive interface. Calcium ions bind to the four interdomain interfaces that separate the five independent cadherin extracellular domains from one another (**Figure1**). The

binding of three calcium ions is made possible by coordination with the carbonyl groups in acidic amino acid groups and sidechain and backbone amides from the linker region and from the segments that connect the beta-strands in the flanking domains. This calcium coordination complex forms when the three calcium ions bind between two cadherin subunits and stabilizes the relative orientation of the extracellular domains. It is important to note that although this conformational alignment of the modular EC domains is often represented as a rod-like shape, it is believed to be a curved shape such that the EC5- end (membrane proximal) and the EC1 end (distal) are oriented approximately 90 degrees from each other.[10] The transition from a globular formation to a rigid, curved structure is easily reversible due to the fact that the calcium binding interactions are reversible as well.[18]

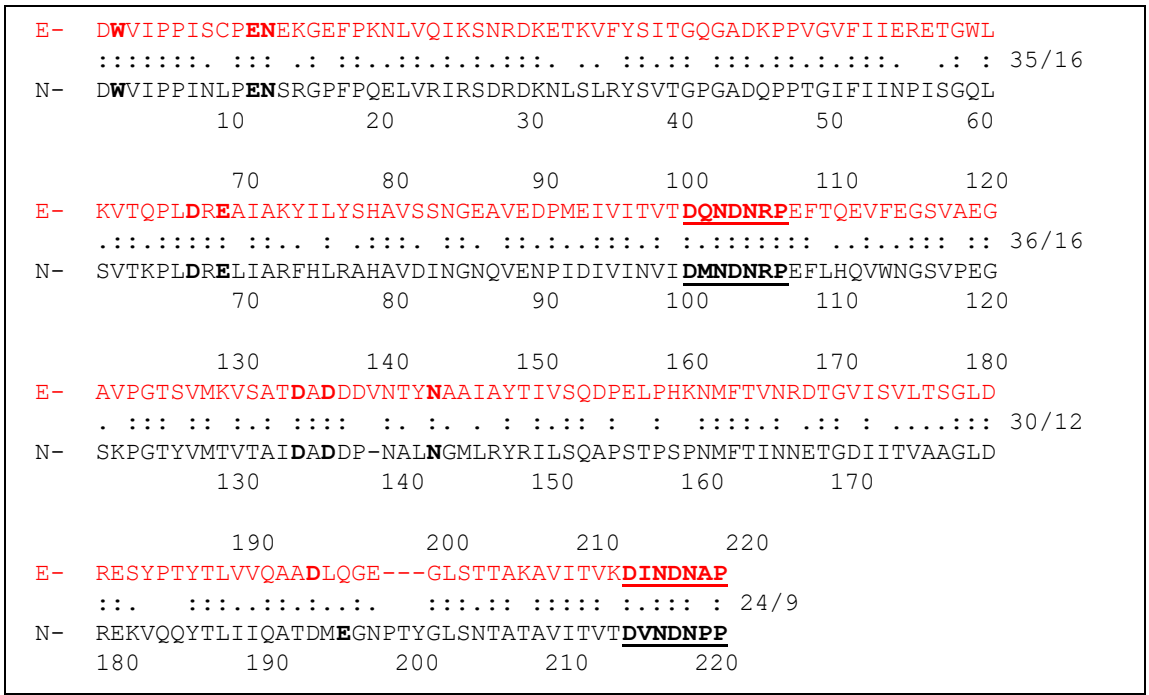

Figure 3: LALIGN comparison of the primary structure of EC12 between E- and N-cadherin.[19]
Amino acids shown in single letter code. W2 is essential for dimerization. E11, N12, D67, E69, D134, D136, N143, D195 are highlighted because they contribute at least one pair of their backbone or side chain electrons to chelate one of the three calcium ions in the interdomain region. The linker segments are underlined and bold. Their consensus sequence is DXNDNXP.

The originally proposed model for trans – dimerization of N-cadherin was that of a zipper adhesion model that contained both cis and trans interactions at the adhesive interface.[9] Cis interactions are between protomers emanating from the same cell surface. Trans interactions are between protomers from apposing cell surfaces. This model has been revised over the following 2 decades, and is summarized in the structures of the 5-domain construct first reported by Boggon, et al.[10] Our interest is in the trans dimer, or the adhesive dimer and is illustrated in the structure of the EC1-EC1interface from Shapiro, et al.[9] **Figure 4** shows a ribbon drawing of a strand-swapped structure in which W2 residues from adhesive partners swap hydrophobic pockets (contains Ile, Ala, Tyr, and the aliphatic portion of Glu) and dock in the partner's pocket. This structure is like a matched pair of similar to that of a "ball and socket" joint.

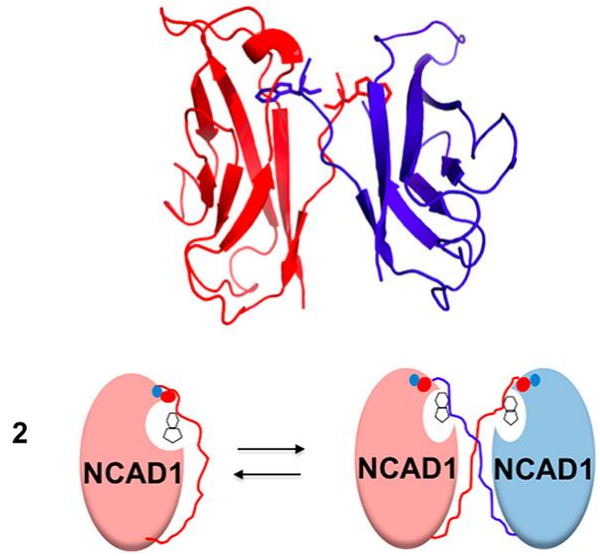

Figure 4. Structure of the strand-swapped interface.

Top figure is the original strand swapped dimer structure.[9] Ribbon drawing was made to highlight the positioning of the side chain of W2 in the hydrophobic pocket of the adhesive partner.[20] The cartoon below illustrates that two "closed" monomers interact to form the strand-swapped dimer. In studies reported in this document, the construct contains EC1 and EC2, and calcium stabilizes the strand-swapped dimer (not shown here).

The binding of calcium stabilizes the strand-swapped dimer. This point was addressed in biophysical studies by Vunnam, et al.[21] In these studies she demonstrated that EC2 in the calcium bound monomeric form was less stable than EC2 calcium bound dimeric form. Two conclusions resulted from this work. First, calcium binding stabilizes the molecule. However,

this net stabilization belies strain in the EC2 domain that is relieved upon strand swapped dimerization. Taken together, one can see that the A-strand is connected to the calcium bound at Site 1 through E11. At the N-terminus it is also tethered to the hydrophobic pocket by docking of W2. Studies by Vendome, et al, (2011) showed that the end-to-end distance of the beta A strand was shorter in the strand-swapped dimer than in the closed monomer, indicating that the closed monomeric structure is extending the beta A strand more than its optimum length.[22]

Our current studies focus on the calcium dependent dimerization of NCAD12, N-cadherin EC1 and EC2. This construct is the minimal functional portion of classical cadherins that binds calcium and dimerizes. We noticed some time ago that N-cadherin will form a dimer that is in slow exchange with monomer, a property that is not shared with E-cadherin. The scenario by which the "trapped" dimer forms is illustrated in the **Figure 5** below. In the presence of 1 mM Ca^{2+}, NCAD12 monomer and dimer are in fast exchange with a $t_{1/2}$ of 15 sec.[23] EDTA is a chelator of many divalent cations, and binds Ca^{2+} with high affinity (K_d ~nM).[24] Addition of EDTA to NCAD12 in 1 mM Ca^{2+} will rapidly depleted calcium from the solution and strip it from the calcium binding sites in the monomeric and dimeric forms of NCAD12. When the calcium binding sites in the D_{sat} dimer are stripped, the dimer is no longer in exchange with monomer and is illustrated in **Figure 5** as D^*_{apo}. In Size Exclusion Chromatography we observe two distinct peaks that allow us to quantitate the concentration of M_{apo} and D^*_{apo}. From those values we calculate a K_d for dimerization. While the simple chromatographic experiment has proven to be a reliable assay for determining K_d for dimerization, we have long speculated on whether this dimer has any physiological relevance.

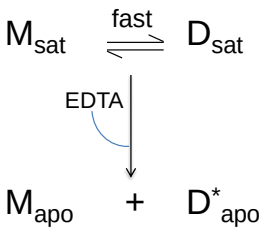

Figure 5: Formation of the "trapped" NCAD12 dimer.

Calcium saturated monomer and dimer are in fast exchange with each other. When calcium is stripped from the solution by addition of EDTA, the rate of exchange between monomer and dimer decreases to the point that a monomer and dimer peak can be distinguished by Size Exclusion Chromatography.

Goal of Current Studies

Neural cadherin has a unique physiological milieu in a developed vertebrate. It is the major cell adhesion protein at neurological synapses, particularly excitatory synapses. The unique property of excitatory synapses is that they connect two neurons, both of which transiently take up extracellular calcium as the signal passes from one neuron to the other. This phenomenon leads to depletion of calcium at excitatory synapses that have been measured to be as low as 40 μM,[16] a value that is 1/25th of the value that is typical of the extracellular space in the rest of the body.[25-27] What happens to the kinetics and equilibria of dimerization at this low calcium concentration? The studies reported here determine the equilibria of dimerization at 40 μM, 100 μM and 1 mM calcium concentrations. Further, we propose a model to explain the difference in the dimerization constants that depends solely on the actual fraction of NCAD12 molecules that are calcium saturated at each of the 3 calcium concentrations.

Methods

Preparation of Protein:

Protein Cloning, expression, and purification of the first two extra cellular segments of the wild type N-cadherin (NCAD12) was accomplished in the same manner as work previously completed in the laboratory and is outlined in previous literature.[28] The NCAD12 unit of N-cadherin consists of amino acid residues 1 through 221 and is minimal functional unit to study the calcium-dependent dimerization of N-cadherin in vitro as it contains EC1, linker region 1, EC 2, and linker region 2. Just as in previous work, the stocks of the NCAD12 protein were prepared via overexpression in bacterial cells and then purified from the inclusion body pellets via denaturing HisTag chromatography.[28] These stocks were stored at -20 ºC in 10 mM HEPES, 140 mM NaCl, pH 7.4 until experimentation began.

Preparation of Buffers:

In order to study the calcium dependence of dimerization of N-cadherin, we prepared buffers of known calcium concentration. The basic characteristics of the buffers were 10 mM HEPES, 140 mM NaCl, pH 7.4. HEPES (FisherSci: PN BP 310) and NaCl (FsherSci: PN S 271) were added as dry crystalline solids. To create the different calcium concentrations, we added the appropriate amount a 1 M $CaCl_2$ standard (Teknova: PN C 0478) to create 40 μM, 100 μM, and 1 mM total calcium in the buffer. The pH of these buffers was adjusted at room temperature with NaOH, but they are currently stored at 4 ºC.

Protein Dialysis:

In order to equilibrate the N-cadherin stock in each buffer, we performed dialysis. Frozen stocks of NCAD12 were thawed. Approximately 0.5 mL of stock was placed in dialysis tubing (Spectra/Por, 6-8 kD MWCO), and then dialyzed against 200 mL in 5 exchanges over a 2-day period at 4 ºC. Post dialysis samples were retrieved from the dialysis tubing, transferred to microcentrifuge tubes, and stored at -20 ºC.

Concentration Determination:

The concentration of NCAD12 within the prepared protein stocks was determined using a NanoDrop absorbance spectrophotometer.[29] Using the Beer-Lambert law, the concentration of each stock was determined using a standard pathlength of 1 cm and the standard molar absorptivity of NCAD12, previously determined to be 15900±400 $L \cdot mol^{-1} \cdot cm^{-1}$.[28] Using the NanoDrop and the Beer-Lambert law, the concentrations of the protein stocks were determined to be $1.14 \cdot 10^{-4}$ M, $9.97 \cdot 10^{-5}$ M, and $8.77 \cdot 10^{-5}$ M for the stocks used with the buffers varying by 1 mM, 100 μM, and 40 μM calcium ion concentrations, respectively.

CD Spectra:

Qualitative structural changes as a function of calcium concentration were assessed in NCAD12 protein using Circular Dichroism (CD) spectroscopy. This technique acquires a spectral signal that measures the difference in absorbance of left and right circularly polarized light. An AVIV 202SF CD spectrometer with CDS 3.02A software was used to acquire the CD spectra for these samples. The use of CD spectroscopy is pertinent to the study of cadherin proteins due to the widely recognized negative signal that the family produces when analyzed via this method.[23] This characteristic negative peak presents itself around 218 nm on any CD

spectrum and is characteristic of the β-sheets present in the extracellular subunits, and indicates that left hand polarized light is absorbed more than right hand polarized light.[30] The CD scans were gathered using a quartz cuvette with a 0.5 mm path length. CD spectra were gathered in 1 nm steps from 300 nm to 200 nm with a 3 s averaging time for each step. The blanks used for our experiments were the prepared buffer solutions at each calcium concentration. Data that are reported below represent the average of 2 CD scans for each sample that were blanked with the corresponding buffer. This methodology allows for comparative analysis of the CD spectra between the protein stock at the varying calcium concentrations of 40 μM, 100 μM, and 1 mM.

HPLC Size Exclusion Chromatography:

In order to determine the fraction of protein in monomeric and dimeric states, Size-Exclusion Chromatography (SEC) was employed to separate monomer from dimer using a High-Performance Liquid Chromatograph (HPLC). All experiments were performed on an Agilent 1100 HPLC Chemstation with UV-Vis absorbance detection at 280 nm (320 nm reference) and a 1 mL/min flow rate. The column was ZORBAX GF-250 analytical column (4.6 x 250 mm 4 Micron, Agilent). The total column volume is approximately 5 mL, and 5 μL of protein was loaded on to the column. All studies were conducted in apo mobile phases (140 mM NaCl, 10 mM HEPES, pH 7.4) at room temperature.

Sample preparation was key to the ability to distinguish monomer and dimer species on the column. In the presence of 1 mM calcium, monomer and dimer are in fast exchange. Thus, we would expect a single broad peak with an elution volume between the dimer elution volume (first eluted peak) and the monomer elution volume (later eluting peak). N-cadherin has a unique property in that when calcium is stripped from the solution, the calcium bound to the protein is rapidly removed, and the dimeric form becomes trapped as a dimer and can be independently

assessed from the monomer using SEC. This phenomenon is well documented by the Pedigo laboratory and was first validated in 2011 by Nagamani Vunnam.[28] Thus, our prepared samples here were treated as follows prior to chromatography. Dialysates were thawed and aliquoted into microcentrifuge tubes. They were incubated at either 4 ºC or at room temperature (~22 ºC) for a fixed period of time noted in the figure legends below. Immediately before analysis, a 10 or 15 μL volume was transferred into a sample vial insert and 1 uL of 0.1 M EDTA was added. The EDTA concentration far exceeded the calcium concentration in the sample, and chelated all free and protein-bound calcium in the sample. After addition the sample was incubated between 20 and 50 minutes as it awaited analysis via the autosampler on the HPLC.

Subsequently, data were exported as a .cvs file format, and parsed into excel. Peak heights were determined by subtracting the baseline (Abs 280 @ 2 min) from the dimer and monomer peak maxima at ~2.3 and ~2.5 minutes, respectively.

Results and Discussion

This portion of the thesis presents and discusses the results of experiments to assess the effect of known levels of free calcium on the structure and function of NCAD12. Spectral data queries the effect of calcium on the structure of NCAD12. Size exclusion chromatographic analysis of EDTA-trapped solutions of NCAD12 at different free calcium concentration allows calculation of the apparent K_d for dimerization. In a subsequent section entitled Theoretical Calculations, we will explore a theoretical model that attempts to explain the calcium-dependence of dimer formation.

CD Spectra:

Circular Dichroism spectroscopy was used as a way of measuring the secondary structural changes of the protein stocks as a result of the varying calcium concentration buffer solutions. These signals were prepared to a standard concentration of NCAD12 (50 μM) to have direct comparison of the absorbance of elliptically-polarized light to compare the magnitudes of the signal. The spectra demonstrate a strong negative CD signal from 220 nm to 207 nm. This is indicative of the beta sheets present in the two modular 7-strand beta sheet domains in NCAD12. The calcium-dependent variance of the magnitude of spectrum is demonstrated in **Figure 6**. As the calcium concentration of the buffer solution increases, the magnitude of the CD spectral signal decreases. Normally, we would expect that the binding of calcium would induce structure

in NCAD12 leading to an increase in negative signal at 215 nm, the typical λ_{max} for beta sheet proteins.[31]

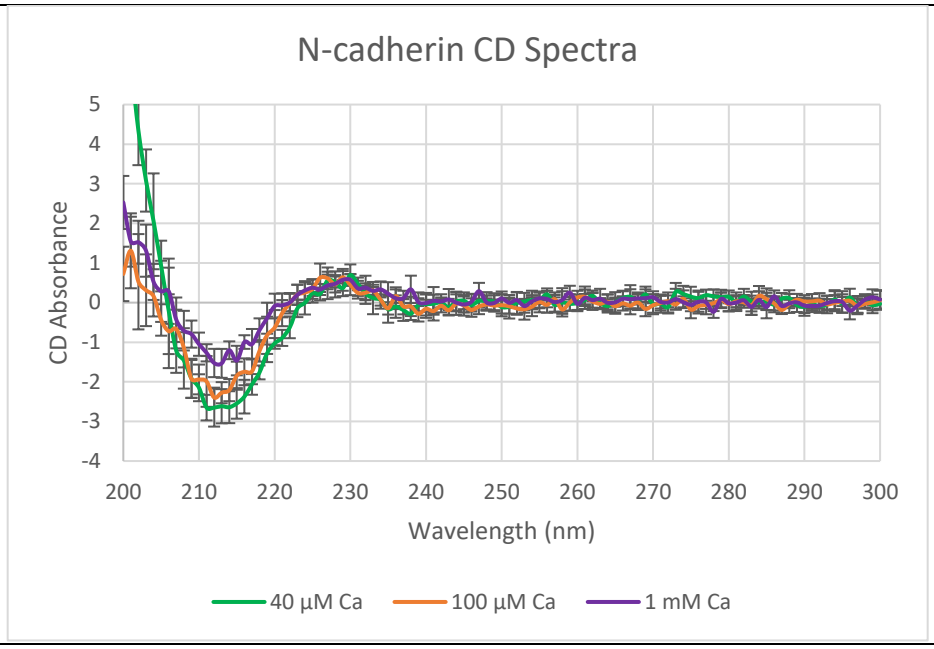

Figure 6: CD spectra of NCAD12 (50 µM) dialyzed in buffers 40 µM, 100 µM or 1 mM Ca^{2+}. Spectra are the average of the spectra from two independently prepared solutions. Spectra were corrected for the buffer and offset at 300 nm.

One of the characteristics of both E- and N-cadherins is that their CD signal becomes more negative as the protein unfolds.[32] These data indicate that contrary to our logical expectation, the signal of E- and N-cadherin will decrease as the domains become more structured. Thus, as calcium binds, the concentration of calcium bound protein will increase, and the structure should increase. This phenomenon is witnessed by the relative decrease in the CD signal at 215 nm as a function of increasing calcium concentration. In summary, the magnitude of the CD signal has an inverse relationship with the calcium concentration present in the buffer solutions used. Further, since the protein concentration is 50 µM, at 1 mM Ca^{2+}, there should be > 50% dimer in sample (K_d for dimerization is ~25 µM[28, 33]). At lower calcium concentration we

would expect less dimer formation. Thus, more negative signal as the concentration of calcium decreases also correlates with a decrease in dimerization.

Chromatographic Data:

In order to investigate the dimerization of NCAD12 as a function of calcium concentration, analytical size exclusion chromatography was used. This technique is often used to separate mixtures of compounds based on their Stokes radius,[34] and an appropriate technique for simple separation of the dimer and monomer forms of NCAD12. This method can determine the equilibrium constant between the two structures within a solution. As given by the equation below, the K_d of the protein is a function of the fraction of monomer and dimer in protein solutions of known concentration.

$$K_d = \frac{[M]^2}{[D]}$$

The key to using this technique is that the monomer and dimer cannot be in fast exchange. If they were in fast exchange, then the protein peak would elute at a weighted average size between the monomer and dimer. Since the technique dilutes the original sample volume, it is difficult to determine the concentration and therefore, to estimate the K_d from this weighted average elution volume. It is important to note that the use of EDTA as a chelator was necessary, as the exchange between monomer and dimer N-cadherin units is in fast exchange in the presence of calcium. In order to acquire an accurate measurement of concentrations of monomer and dimer, it was necessary to chelate all free calcium ions in solution in order to stop this rapid exchange.

Figure 7 is an example of one of the chromatograms resulting from our study. As shown, the two chromatographic peaks at 2.3 min is dimer, and at 2.5 min is monomer. The large peak at 3.0 min is from the EDTA that was added to "trap" the dimeric form prior to injecting the

solution onto the column. The K_d for this sample can then be determined from the mole fraction of protein that is dimer and is monomer via the ratio of peak height of the dimer and monomer shown in the chromatogram. Note that the fraction dimer is in terms of the monomeric units since the extinction coefficient of the dimer is twice that of the monomer. The values of [M] and [D] in the equation above are determined based on the actual protein concentration in the experiment, and used to calculate K_d, which is a concentration independent value.

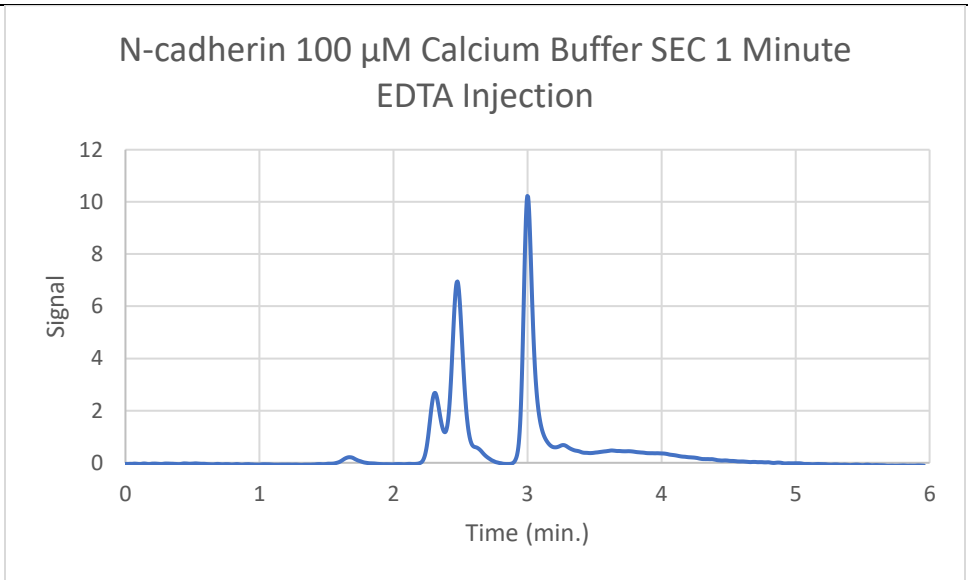

Figure 7: Example chromatogram produced from the size exclusion analysis of NCAD12. Conditions: NCAD12 in100 µM calcium standard buffer solution for 1 minute prior to injection of 0.1 M EDTA (1/15 dilution; 7 mM final concentration). Dimerized NCAD12 (2.3 min) elutes prior to monomer (2.5 min). In this sample, the presence of monomer is much more prevalent than the dimer species.

Dimerization studies were carried out from different protein stocks, at different protein concentrations and at different temperatures. **Table 1** is the collective results from the analysis of all chromatographic data from trapped NCAD12. As illustrated in **Table 1**, there is a stark difference in the dissociation constants of the N-cadherin protein units in relation to the concentration of calcium ions within the standardized buffer solutions. As the calcium concentration decreased, the concentration at which half of the protein was in the dimeric state

increased from ~ 30 μM (1 mM Ca^{2+}) to ~ 340 μM (40 μM Ca^{2+}). This result indicates that the apparent dimerization affinity is decreased by more than a factor of 10 and the calcium concentration decreases from 1 mM to 0.04 mM at 4 °C.

[Ca^{2+}]	Temperature (°C)	Average apparent K_d (μM)	Number of Trials
1 mM	25	26 ± 6	2
100 μM	25	106 ± 17	2
1 mM	4	31 ±9	4
100 μM	4	88 ± 15	5
40 μM	4	342 ± 19	3

Table 1: Summary of K_d values determined from chromatographic studies.
Note: The larger the value of K_d, the lower the affinity of dimerization.

One important goal of this study is the use of two different temperatures in order to investigate the contribution of enthalpy to dimerization of NCAD12. In order to investigate this phenomenon, solutions to be injected into the chromatograph for analysis of protein were prepared at both 4 °C and 25 °C. In order to perform the analysis, the samples that were prepared at room temperature were taken out of the refrigerator (4 °C) and placed on a lab bench for one week in order to guarantee that the monomeric and dimeric forms equilibrated at 25 °C. When the data were analyzed at these two preparation temperatures, there was no statistical difference between the values of the dissociation constants. This indicates that there is little enthalpic effect on the dimerization of N-cadherin at 1 mM and 100 μM calcium ion concentration. This observation could not be extended to data for the protein in a 40 μM calcium buffer, as the samples that had been set out to equilibrate at room temperature for a week prior to experimentation had denatured or had been degraded in low levels of calcium. Also note that we

had intended to study the kinetics of dimerization, but were forced to abbreviate experiments due to the closing of the university in mid-March.

Table 1 also illustrates that the dissociation constants of N-cadherin are extremely dependent upon calcium concentration of solution, regardless of the enthalpic effects. As illustrated, the formation of dimer in solutions with higher calcium concentration of 1 mM calcium was experimentally determined to be much higher affinity than in the lower concentrations of 100 μM and 40 μM calcium. This observation is due to lower saturation of the calcium binding sites in the linker region between EC1 and EC2 of the N-cadherin dimer forming interface. Due to the lower calcium concentration, it is much less likely that all three calcium binding sites in this region will be saturated with calcium ions in any given protein unit, thus there is less formation of the dimeric protein species in solution, as more of the protein cannot physically enter into the dimeric state with a partner protein moiety. This phenomenon is addressed in the following section entitled Theoretical Calculations.

Theoretical Calculations

Fractional saturation of the calcium binding sites:

As a determined in the size exclusion study, the fraction of the N-cadherin molecules in monomeric and dimeric states is related to the fractional saturation of the three calcium ion binding sites located in the linker region between EC1 and EC2 in each N-cadherin protein unit. If these sites do not contain a chelated Ca^{2+} ion within them, then processes necessary for dimerization, such as the imbedding of each unit's W2 residue into the partner unit's hydrophobic pocket, will not proceed, thus leading to less dimer formation. For this study, calcium ion concentrations of 40 μM, 100 μM, and 1 mM were chosen as an experimental model of the calcium concentration gradient present within the body. The 1 mM calcium concentration buffer solution serves as the representation for the environment of the extracellular fluid within the body. N-cadherin is not found in this region of the body, as the protein resides within the synaptic cleft of neurons, a segregated space that varies drastically from the environment of the extracellular fluid. The synapses are isolated from the high calcium concentrations of the rest of the body by the blood-brain barrier, but also by the compartmentalization of space by glial cells that support the neurons. In all, the brain and excitatory synapses in particular are diffusion restricted space through which the concentration of ions differs from the EC-space in general. Due to this impermeable structure, the calcium ion concentration for the environment in which N-cadherin operates is much lower than 1 mM and may undergo transient drops due to neurological signal transduction across the synapse. The calcium concentration at excitatory synapses has been measured to be between 40 and 100 μM, values that are 1/25 and 1/10 the value of the concentration in the EC-space in general. To study dimerization at this lower

physiological Ca^{2+} concentration, it was necessary to use a lower calcium ion concentration, including 40 µM Ca^{2+} and 100 µM Ca^{2+} to study the level of dimer. Both of these Ca^{2+} concentrations serve as a counterpoint to the experimental reference used to monitor the amount of dimerization of the N-cadherin protein in more typical studies. With the data from the reference (1 mM) and test (40 and 100 µM) calcium concentrations, the effects of the fractional saturation of the three calcium binding sites within the linker region between EC1 and EC2 was examined. The saturation of the sites was modeled as shown in **Figure C**.

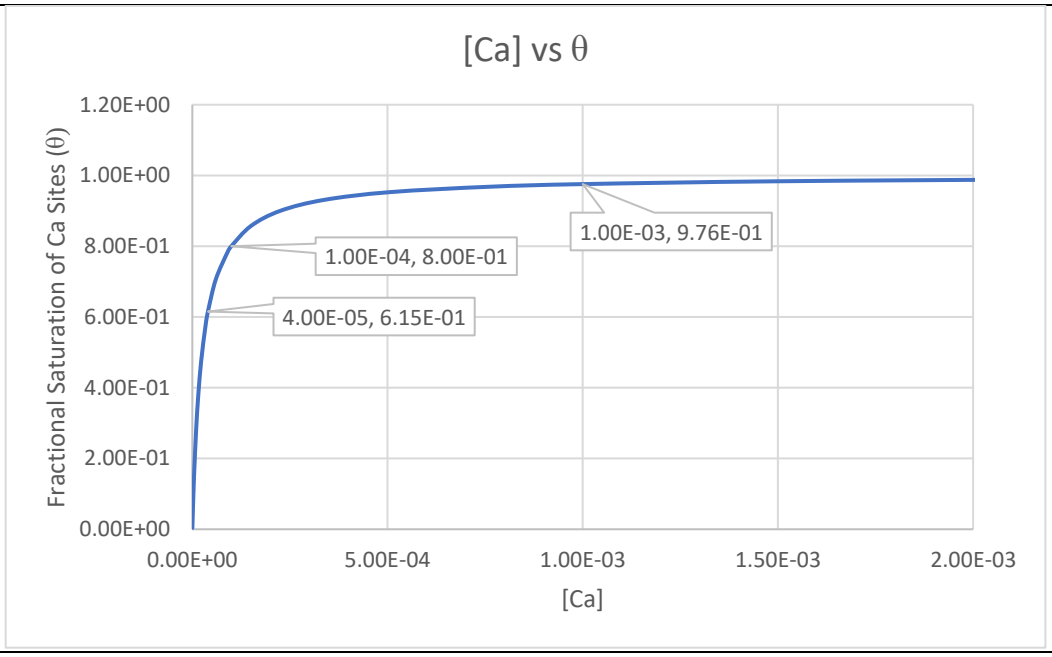

Figure 8: Simulated data of a calcium binding curve for N-cadherin.
Graphical representation of the relationship between calcium ion concentration (x-axis) and the fractional saturation of the calcium ion binding sites in the linker region between EC1 and EC2 of N-cadherin proteins (y-axis). The percentage of binding site saturation was found using the documented K_a for Ca^{2+} binding to such sites.

Fractional saturation was simulated based on the following equation

$$\theta = \frac{X}{K_d + X} = \frac{KX}{1 + KX}$$

where X is the calcium concentration and K_d is the dissociation constant for calcium, a value determined to be 25 μM.[35] Notice that at a calcium concentration four times the K_d for calcium, the protein is 80% saturated on average. Notice that at 1 mM calcium, the calcium binding sites are 97.8 % saturated on average. Finally, at only 40 μM calcium, the calcium concentration is approximately the value of the K_d. As we would expect, the calcium binding constants should be just above half saturation, and we calculate that value to be 61.5% saturated.

Our system is more complex since 3 calcium ions bind to each molecule, not just one. Thus, using this equation to describe calcium binding to NCAD12 requires several important assumptions. First, we assume that the free calcium concentration is known. This assumption is valid since we dialyzed the apo stock of N-cadherin against these buffers to set up the equilibrium. Second, we assume that the calcium binding sites have equivalent affinity. If they have equivalent affinity, and the solution is equilibrated at say 100 μM Ca^{2+}, then we would expect that all sites will be occupied at 80%, and 20% of the sites are empty. What we do not know is which and how many of these sites are empty.

To determine the concentration of fully saturated Monomer (M_3), we can do the simple calculation outlined below where M_1 has one ligand bound, M_2 has two ligands bound, and M_3 has 3 ligands bound. Y is the saturation of sites, so for our system would be a maximum value of 3.

$$Y = \frac{M_1 + 2M_2 + 3M_3}{M_o + M_1 + M_2 + M_3}$$

$M + X \leftrightarrow M_1$; $K_1 = M_1/M_o{*}X$; $M_1 = K_1M_oX$

$M + 2X \leftrightarrow M_2$; $K_2 = M_2/M_o{*}X^2$; $M_2 = K_2M_oX^2$

$M + 3X \leftrightarrow M_3$; $K_3 = M_3/M_o{*}X^3$; $M_3 = K_3M_oX^3$

$$Y = \frac{K_1M_oX + 2K_2M_oX^2 + 3K_3M_oX^3}{M_o + K_1M_oX + K_2M_oX^2 + K_3M_oX^3}$$

$$Y = \frac{K_1X + 2K_2X^2 + 3K_3X^3}{1 + K_1X + K_2X^2 + K_3X^3}$$

If sites are equal and independent, then $K_1 = 3K$; $K_2 = 3K^2$; $K_3 = K^3$, where K is the association constant for the equal and independent sites. The multipliers subsumed in K_1 and K_2 result from the statistical factors for the number of ways in which the intermediate states can form. They are only one way to make empty and full and three possible ways to form the species with one empty or 1 full site (Pascal's triangle or Fibonacci Series).

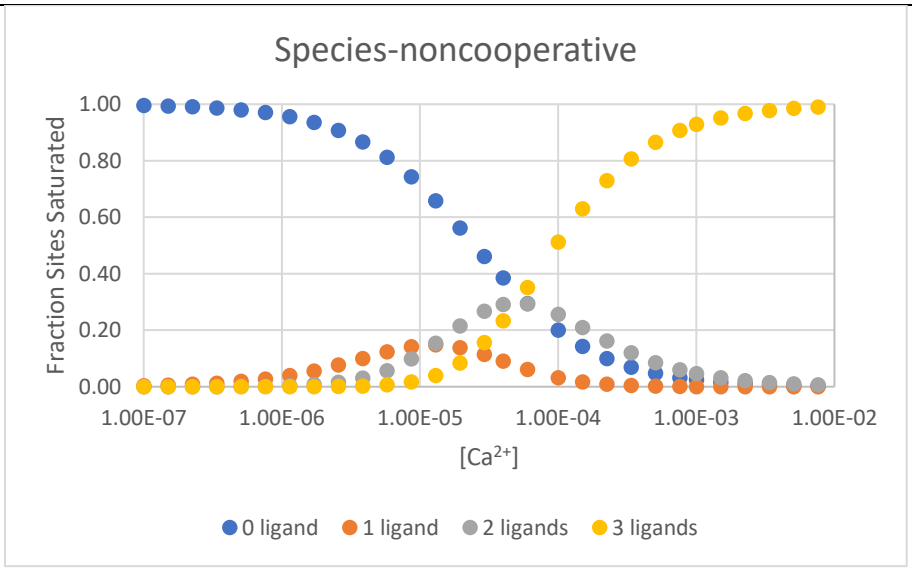

Figure 9: Simulation of the abundance of species as a function of [Ca^{2+}] assuming equal and independent sites.
Apo (blue), 1 ligand bound (orange), 2 ligands bound (gray) and saturated (yellow) change in abundance as a function of calcium concentration (x-axis).

[Ca^{2+}], µM	Equal and independent
40	0.233
100	0.512
1000	0.929

Table 2: Predicted fraction of total protein that is calcium saturated at each calcium concentration.

In the next section, we will revise our assessment of the dimerization constants based on the total protein concentration to take into account how these different models for calcium binding impact the estimation of K_d for dimerization.

Equilibrium constant dimerization as a function of calcium concentration:

The purpose behind this section is to first simulate the expected level of dimer based on the values for K_d of dimerization reported in **Table 1** in the Results section above. To do this, we simulated the fraction dimer as a function of total protein concentration for each of the calcium concentrations. For the dissociation of dimer, the K_d expression is in the form of a quadratic equation, which we solve for [M]. We report the concentration of dimer in terms of monomer ($[D]_{itom}$) since it is the form of the value resulting from SEC studies.

$$D \leftrightarrow M + M;\ K_d = \frac{[M]^2}{[D]};\ \text{Total} = M + 2D$$

$$[M] = \frac{-K_d + \sqrt{K_d{}^2 + (4 \times 2 \times K_d \times Tot}}{2 \times 2}$$

$$[D]_{itom} = Tot - [M]$$

In **Figure 10** below, we see the result of that simulation. In every case, as the concentration of NCAD12 increases, the level of dimer in the solution increases as we would expect from simple mass action for the formation of dimer. Next, notice that as the calcium concentration decreases, the midpoint of the curves moves toward the right, or toward higher protein concentrations. This indicates that at lower calcium concentrations, the amount of dimer formed at a particular protein concentration decrease. Conversely, the amount of protein required to form 50% dimer in solution increases as the calcium concentration in the solution decreases.

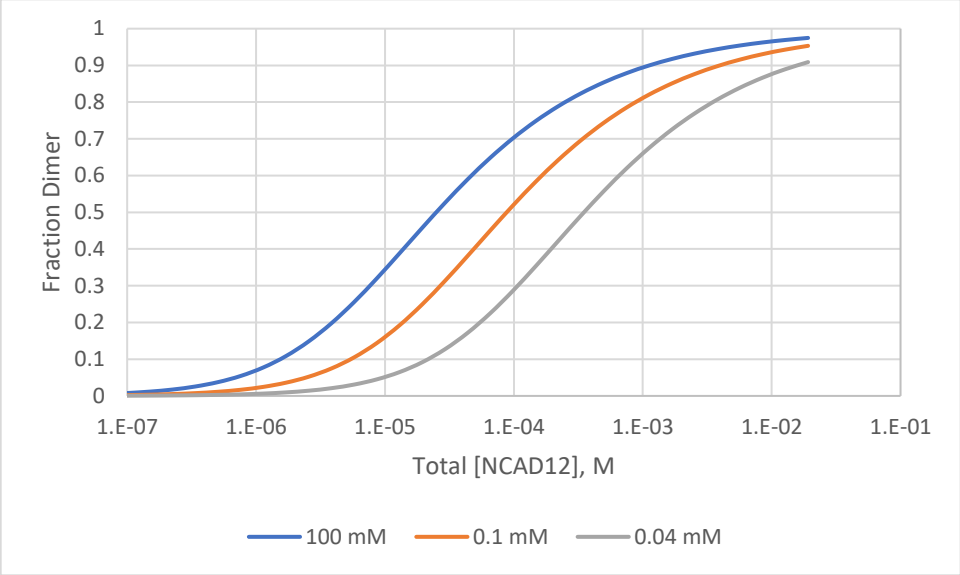

Figure 10: Simulated data to illustrate the effect of free calcium on the formation of NCAD12 dimers.

The protein stocks under study here are all approximately 100 μM. Drawing a vertical line at the 1e-4 M (x-axis) intersects with the 1 mM curve (blue) at 0.7, with the 0.1 mM curve (orange) at ~ 0.5 and with the 0.04 mM curve (gray) at ~ 0.25. These values are almost exactly equivalent to the fraction dimer found in the chromatography experiments reported in **Table 1** in the Results section. This observation is important as a bench mark to validate the simulations.

Note that the K_d values used in this simulation assume all of the protein in solution is competent to form dimer whether it is calcium saturated or not. We are certain that this is a gross overestimation of the dimer-competent protein concentration since calcium-bound forms are required for dimerization. Now we will use the fractional saturation data from the calcium binding simulations in the previous section to "recalculate" the values of K_d at each calcium concentration based on the model-dependent prediction of the concentration of calcium-saturated species in the table below.

$[Ca^{2+}]$, mM	K_{dTot} μM	f_{sat} (+++)	K_{d+++} μM	$f_{sat\ Eq\&Ind}$	$K_{d(EqInd)}$ μM
0.040	350	0.615	214	0.233	81
0.100	88	0.800	71	0.512	46
1.00	31.5	0.976	31	0.929	29

Table 3: Simulation K_d recalculation and comparison

K_{dTot} (grey) is calculated based on total protein saturation with calcium, as proposed by **Table 1**. K_{d+++} (orange) incorporates infinite cooperativity of calcium binding with the fractional saturation of sites from **Figure 8**. $K_{d(EqInd)}$ (green) represents the equal affinity binding of calcium with no cooperativity as well as the possibility of intermediate saturation states, as in **Figure 9**.

In the above table, K_{dTot} is calculated based on the assumption that all protein is saturated with calcium and is able to undergo dimerization. K_{d+++} is calculated based on the assumption that the binding of calcium is infinitely cooperative such that the fractional saturation of sites ($f_{sat(+++)}$) is equivalent to the fraction of total protein that has all three calcium ions bound. $K_{d(EqInd)}$ is calculated based on the assumption that the calcium binding sites have equal affinity and no cooperative interactions. The fractional saturation of sites ($f_{sat(Eq\&Ind)}$) is somewhat less than the previous model since intermediate states can be populated as shown in **Figure 9**.

First, let us consider the data for K_d in 1 mM Ca^{2+}. The value for K_d is ~ 30 μM since the fraction of molecules with all three calcium ions bound is ≥ 0.929, so very close to completely saturated. If we consider this as the value for K_d when all sites are saturated, it becomes the benchmark for comparison to data at lower calcium concentrations. In the subsequent two columns, the calculations assume infinite cooperativity. Therefore, the level of dimer-competent protein is directly determined by the fractional saturation of sites. In the orange-shaded columns as we work from high calcium to low calcium levels, the values for K_d decrease compared to the

K_{dTot} values indicating that the binding must be higher affinity if the amount of observed dimer was actually formed by a smaller fraction of the total protein. A similar effect is witnessed for the predicted K_d based on the values of calcium saturated dimer predicted from the equal and independent sites binding model.

The message from this model dependent analysis of the linkage between calcium binding and dimerization indicates that the data converges on the "benchmark value" of ~30 μM. These data indicate the utility of simulating fundamental phenomena in thermodynamic linkages such as calcium dependent dimerization of cadherins. These studies will serve as a basis for future work in which we can explore calcium binding models that more closely model sequential binding and populated intermediate states.[35]

Conclusion

In this study, instrumental analysis, circular dichroism spectroscopy and size exclusion chromatography, was partnered with theoretical calculations in order to better understand the dimerization model and equilibrium states of N-cadherin within its unique physiological environment of low calcium concentration. The studies reported here determine the equilibria of dimerization at 40 μM, 100 μM, and 1 mM calcium in order to allow for a comparison of the protein dimerization equilibria in the different calcium environments of the body. Further, the study proposes a model to explain the difference in the dimerization constants that depends solely on the actual fraction of NCAD12 molecules that are calcium saturated at each of the 3 calcium concentrations. From the derivation of this model, further experiments can be performed that may further illuminate the mechanism of calcium dependent dimerization of neural cadherins.

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