Nickel Reduces Calcium Dependent Dimerization In Neural Cadherin

Matthew Paul Dukes

University of Mississippi

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ABSTRACT

Cadherins are the primary transmembrane component in adherens junctions, structures that link the actin cytoskeleton in adjacent cells within solid tissues including neurological synapses, epithelium and endothelium. Cell-cell adhesion by cadherins requires the binding of calcium ions to specific sites in the extracellular region. Given the complexity of the cell adhesion microenvironment, we are investigating whether other divalent cations might affect calcium-dependent dimerization of neural-(N-) cadherin. The first chapter focuses on studies to characterize the impact of binding physiological magnesium (II) or neurotoxic nickel (II) on calcium-dependent N-cadherin function. Physiological levels of magnesium have only a small effect on the calcium-binding affinity and calcium-induced dimerization of N-cadherin. However, a tenfold lower concentration of nickel decreases the apparent calcium-binding affinity and calcium-induced dimerization of N-cadherin. Competitive binding studies indicate that the apparent dissociation constants for nickel and magnesium are 0.2 mM and 2.5 mM, respectively. These Kd values are consistent with concentrations observed for a range of divalent cations in the extracellular space. Results from these studies indicate that calcium-induced dimerization by N-cadherin is attenuated by natural and non-physiological divalent cations in the extracellular microenvironment. The second chapter will detail my contributions to studies into the effect of pH on the structure and function of neural cadherin that utilize many of the same analytical techniques.
DEDICATION

This work is dedicated to my wife Emily, without whose tireless encouragement and support I would have given up long ago.
# LIST OF ABBREVIATIONS AND SYMBOLS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Apo</td>
<td>Calcium-depleted</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>EC</td>
<td>Extracellular domains</td>
</tr>
<tr>
<td>EC1</td>
<td>Extracellular domain 1 of NCAD12</td>
</tr>
<tr>
<td>EC2</td>
<td>Extracellular domain 2 of NCAD12</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>GHCl</td>
<td>Guanidinium Hydrochloride</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>K_d</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>NCAD12</td>
<td>Neural-cadherin domains 1 and 2 (residues 1 to 221)</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type NCAD12</td>
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</table>
ACKNOWLEDGMENTS

This body of work would not have been possible without insight, support, and guidance from so many of the helpful students and professors in the Department of Chemistry and Biochemistry:

- My advisor, Dr. Susan Pedigo
- My committee members, Drs. Godfrey and Watkins
- My graduate advisor, Dr. Walt Cleland
- My labmates, Chris Fox, Samantha Davila, and Xiaoyun Howard
- All of the undergraduate students who have contributed to these studies over the years
# TABLE OF CONTENTS

ABSTRACT .......................................................................................................................... ii
DEDICATION ......................................................................................................................... iii
LIST OF ABBREVIATIONS AND SYMBOLS ....................................................................... iv
ACKNOWLEDGMENTS .......................................................................................................... v
LIST OF TABLES ................................................................................................................... vii
LIST OF FIGURES ................................................................................................................ viii
CHAPTER I ......................................................................................................................... 1
   INTRODUCTION ................................................................................................................ 1
   METHODS ............................................................................................................................ 5
   RESULTS ............................................................................................................................ 8
   DISCUSSION .................................................................................................................... 13
CHAPTER II .................................................................................................................... 18
   INTRODUCTION ................................................................................................................ 18
   METHODOLOGY ............................................................................................................. 22
   RESULTS ............................................................................................................................ 25
   DISCUSSION .................................................................................................................... 29
REFERENCES ..................................................................................................................... 32
VITA .................................................................................................................................. 43
LIST OF TABLES

Table 1 ........................................................................................................................................... 9
Table 2 ........................................................................................................................................... 11
Table 3 ........................................................................................................................................... 25
Table 4 ........................................................................................................................................... 28
LIST OF FIGURES

Figure 1 ............................................................................................................................. 8
Figure 2 ............................................................................................................................. 10
Figure 3 ............................................................................................................................. 12
Figure 4 ............................................................................................................................. 15
Figure 5 ............................................................................................................................. 26
Figure 6 ............................................................................................................................. 27
CHAPTER I

NICKEL REDUCES CALCIUM DEPENDENT DIMERIZATION IN NEURAL CADHERIN

INTRODUCTION

As the primary transmembrane component of adherens junctions, cadherins mediate calcium-dependent cell-cell adhesion. Calcium binds to the extracellular region at sites that comprise clusters of negatively charged residues at the interface between each of the five tandem modular extracellular domains. Given the itinerant nature of the calcium binding sites in cadherins in comparison to EF-hand calcium binding sites[1] or Zn-finger sites,[2] we might expect that other divalent cations would bind to cadherin and compete with calcium for occupancy of the calcium-binding sites. This paper addresses three related issues: do alternative divalent cations compete with calcium binding; can their binding induce dimer formation; and does competition with calcium binding affect adhesive dimer formation by N-cadherin (NCAD). Studies focus on the effect of two divalent cations, a naturally occurring ion (Mg$^{2+}$), and a neurotoxic, environmental hazard, (Ni$^{2+}$).

N-cadherin is responsible for mediating a variety of specific cell-cell adhesive events through calcium-dependent dimerization of identical proteins on adjoining synaptic membranes.[3-6] It is involved in a variety of morphological and developmental processes such as synapse formation and maintenance, neurulation, and neurite outgrowth.[7-10] Because these
morphological processes have been shown to occur concurrently with changes in cadherin expression, regulated modifications of the adhesive strength and selectivity of these cell adhesion molecules is critical for proper embryonic development.[11] Cell adhesion molecules, including N-cadherin, are involved in the dynamics and regulation of synaptic structure and function that directly impact induction of long-term potentiation.[12-16] Given the importance of N-cadherin in these physiological processes, it is important to understand the microenvironmental factors that influence its adhesive behavior.

N-cadherin is a classical or type I cadherin comprising five tandem extracellular (EC) domains, a single-pass transmembrane region, and a conserved C-terminal cytoplasmic region. Formation of adhesive dimers between cadherins on apposed cell surfaces occurs through the formation of a strand-swapped structure. This end state forms via the exchange of the N-terminal β-strand between juxtaposed EC1 domains resulting in the mutual insertion of the side chain of a conserved tryptophan (Trp2) into the conserved hydrophobic pocket of the neighboring molecule.[4, 6, 17] Because the critical information necessary for formation and dynamics of cadherin-based adhesion resides in the primary structure of the EC region, the studies in this paper utilize a highly-studied construct possessing only the first two domains of the extracellular region of N-cadherin, NCAD12.[18]

The interface between tandem EC domains contains clusters of carboxylates that constitute three calcium-binding sites. Binding of three calcium ions at these interdomain sites causes a conformational change resulting in a rigidified EC region with an elongated, curved structure.[19-21] Because the residues comprising the calcium-binding sites are anionic, we hypothesized that the presence of other divalent cations in the extracellular microenvironment will compete with calcium for site occupancy, resulting in a reduction of calcium-binding
affinity with a concomittant decrease in dimer formation. Studies, herein, report the effect that Ni\(^{2+}\) and Mg\(^{2+}\) have on calcium-dependent dimerization of N-cadherin.

Cations reside in varying concentrations within the neural extracellular matrix, and are thought to play important roles in modulating neuronal structure and function.[22, 23] Previous work from our lab demonstrated that adhesive dimer formation by cadherin is systematically increased as the concentration of NaCl increases from 60 mM to 1 M, indicating that there is an attenuation of affinity by electrostatic repulsion.[24] While the origin of this electrostatic effect is unknown, it does indicate the critical role of ionic interactions between protomers at the dimerization interface.

Magnesium is an essential nutrient in every cell type in the human body and is found in concentrations of up to 1.5 mM within the extracellular matrix.[25] It participates in a number of physiological processes including signaling, enzyme activation, and catalysis.[26] Magnesium is also the most abundant free divalent cation in cells, further demonstrating its ubiquity in physiological processes.[27] Contrastingly, nickel is known to be a carcinogen, though the molecular mechanism by which it confers this risk is unknown.[28] While nickel possesses an essential role in many physiological processes,[29, 30] the rising consumption of nickel-containing products results in environmental pollution by nickel and its by-products at all stages of production, recycling and disposal.[31, 32] Human exposure to nickel primarily occurs by inhalation and ingestion, and excessive amounts of nickel in different forms may be deposited in the human body over time, by means of both occupational and dietary exposure.[33, 34]

Because the chemistry of the extracellular matrix has numerous implications in the fields of brain physiology and pathophysiology,[35-37] we explored the effects of the Ni\(^{2+}\) and Mg\(^{2+}\) on the biophysical properties of N-cadherin. While these studies show that several aspects of its
function are weakly affected by the presence of Mg$^{2+}$, the presence of Ni$^{2+}$ strongly affected both calcium-binding and dimerization affinities. These results suggest that the presence of alternative divalent cations within the neural extracellular matrix may modulate synaptic dynamics and efficacy.
METHODS

Protein Expression and Purification

The cloning of the gene for the first two extracellular domains (residues 1-221) of NCAD12 (EC1, linker1, EC2, and linker2) was described previously.[38] Briefly, recombinant pET30 Xa/LIC plasmids were amplified by KOD HiFi DNA Polymerase (Stratagene) and transformed into *Escherichia coli* BL21 (DE3) cells. Protein was overexpressed and purified. Protein purity was verified via SDS-PAGE in 17% Tris-Glycine gels through standard protocols. The concentration of the protein stocks was determined spectrophotometrically ($\varepsilon_{280} = 15,900 \pm 400 \text{ M}^{-1}\text{cm}^{-1}$).

Divalent Cation Binding Studies

The circular dichroism signal of NCAD12 was monitored during titrations with Ca$^{2+}$, Mg$^{2+}$ and Ni$^{2+}$ using an AVIV 202SF Circular Dichroism (CD) Spectrometer. For all titrations, solutions of 1 mM, 10 mM, 100 mM, and 700 mM of metal (II) choride were each added sequentially in 2.5 µL, 5.0 µL, and 10.0 µL increments to a 5 µM solution of NCAD12. Titrations were performed in triplicate. CD signal was recorded in wavelength scans from 300-210 nm with an averaging time of 30 seconds. Titration data were considered at a fixed wavelength and then fit to an equation for equal and independent sites with linear apo and saturated baselines.[38] The Ni$^{2+}$ and Mg$^{2+}$ titrations did not yield clear transitions with saturated baselines. As such, they could not be analyzed for binding constants directly. In order to estimate the binding affinities of Mg$^{2+}$ and Ni$^{2+}$, we performed competitive-binding titrations, in which 5 µM NCAD12 containing fixed concentrations of the Mg$^{2+}$ and Ni$^{2+}$ was incubated for five minutes at room temperature, and then titrated with calcium as described above. Binding affinities were resolved from simultaneous analysis of all experiments by fitting to the following
competitive binding model:

\[
\frac{K_x \cdot X \cdot M_o \cdot \left(1 - \frac{K_y \cdot Y}{1 + K_y \cdot Y}\right)}{1 + K_x \cdot X \cdot \left(1 - \frac{K_y \cdot Y}{1 + K_y \cdot Y}\right)} = MX
\]

This model was derived based on the assumption that all 3 calcium binding sites at the interface between EC1 and EC2 are equal affinity and independent of each other. Calcium binding data did not support a more complex binding model.

**Disassembly and Assembly Studies: Analytical Size Exclusion Chromatography**

The impact of Ca\(^{2+}\), Mg\(^{2+}\) and Ni\(^{2+}\) binding on dimer formation was investigated using analytical Size Exclusion Chromatography (SEC). A Superose-12 10/300 GL column (Amersham) was used on an ÄKTA Purifier HPLC system (Amersham) with UV absorbance detection at 280 nm, a 0.5 mL/min flow rate, and a 50 µL injection volume. The mobile phase consisted of 10 mM HEPES, 140 mM NaCl at pH 7.4. Two columns were used over the course of these experiments leading to small differences in elution volume for the SEC data presented here.

**Assembly Studies:** In order to assess the effects of divalent cation on the assembly of dimer, we exploited an analytically useful property of NCAD12. Rapid decalcification of the calcium-saturated NCAD12 dimer (D\(_{\text{sat}}\)) results in the formation of a kinetically-trapped dimer (D\(^*\)\(_{\text{apo}}\)); the concentration of D\(^*\)\(_{\text{apo}}\) represents the amount of D\(_{\text{sat}}\) in the calcium-saturated solution prior to decalcification.[39] In order to test the ability of divalent cation to promote dimer formation, we added cation at a particular level to the apo sample to promote the formation of D\(_{\text{sat}}\). In this case, the 25 µM apo protein samples were exposed to 1.5 mM MgCl\(_2\) or 1 mM NiCl\(_2\) to promote the formation of D\(_{\text{sat}}\), EDTA was added to a final concentration of at least five fold the concentration of cation before the sample was analyzed by SEC. Addition of EDTA depleted divalent cations
in solution and converted $D_{\text{sat}}$ to $D_{\text{apo}}^{*}$ for the purpose of analysis by SEC. The samples were subsequently injected on the SEC column and the level of monomer and dimer was monitored as the height of the peaks detected at 280 nm.

**Disassembly Experiments:** To determine whether divalent cations can disassemble preformed $D_{\text{apo}}^{*}$, we employed an apo protein stock that was previously prepared with a high level of $D_{\text{apo}}^{*}$. Aliquots of the 80 µM apo stock were exposed to 1 mM Ca$^{2+}$, Mg$^{2+}$ and Ni$^{2+}$ for five minutes, and then the level of $D_{\text{apo}}^{*}$ remaining in the stock was determined using the SEC method described above.

**Competition Experiments:** Finally, competitive binding studies were performed as follows. Fixed concentrations of NiCl$_2$ and MgCl$_2$ were added to aliquots of an apo NCAD12 stock (25 µM) and incubated at room temperature for five minutes. Next, 1 mM calcium was added and incubated for an additional five minutes, followed by 5 mM EDTA to form the $D_{\text{apo}}^{*}$ species for analysis by SEC.
RESULTS

The experiments reported in this thesis addressed three related questions: does Mg$^{2+}$ or Ni$^{2+}$ compete with Ca$^{2+}$ for occupancy of the calcium-binding sites; does Mg$^{2+}$ or Ni$^{2+}$ binding induce dimer formation; and does competition with calcium binding affect adhesive dimer formation by N-cadherin. We present data that show while Mg$^{2+}$ and Ni$^{2+}$ compete with calcium binding, they do not induce formation of adhesive dimer directly. However, Ni$^{2+}$ competition with calcium binding strongly decreases calcium-induced dimerization, while the presence of Mg$^{2+}$ has only a small effect. These studies illustrate the importance of considering the complexity of the extracellular microenvironment in which cadherin-mediated adhesion occurs.

Binding of Divalent Cation

Changes in the CD spectra were monitored during calcium titrations of NCAD12 in the absence and presence of fixed concentrations of Ni$^{2+}$ and Mg$^{2+}$. Representative calcium titrations are shown in Figure 1.

![Figure 1](image-url)
As the concentration of Ni\textsuperscript{2+} and Mg\textsuperscript{2+} increased, the midpoint of the titration curve shifted to higher calcium concentration, especially in the presence of Ni\textsuperscript{2+}, indicating a lower apparent calcium-binding affinity as a function of increasing divalent cation concentration. These results imply that there is competition between calcium and other divalent cations in the neural extracellular matrix for the same binding sites in N-cadherin. In order to determine the apparent binding constants for Ni\textsuperscript{2+} and Mg\textsuperscript{2+}, competitive-binding data were fit to Eq 1. Resolved parameters for Ca\textsuperscript{2+}, Ni\textsuperscript{2+} and Mg\textsuperscript{2+} affinity are shown in Table 1.

<table>
<thead>
<tr>
<th>Independent Cation</th>
<th>Ke(Ca\textsuperscript{2+})</th>
<th>18 ± 4x10\textsuperscript{3}</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Competitive Titration</th>
<th>Ke(Ca\textsuperscript{2+})</th>
<th>20 ± 4x10\textsuperscript{3}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ke(Ni\textsuperscript{2+})</td>
<td>5 ± 2x10\textsuperscript{3}</td>
</tr>
<tr>
<td></td>
<td>Ke(Mg\textsuperscript{2+})</td>
<td>4 ± 3x10\textsuperscript{2}</td>
</tr>
</tbody>
</table>

**Table 1.** Association constants for calcium binding monitored by circular dichroism

The resolved association constants for calcium binding in the absence of Ni\textsuperscript{2+} and Mg\textsuperscript{2+} are also shown for comparison. Ni\textsuperscript{2+} binds with an apparent dissociation constant of 200 µM, 4 fold weaker binding affinity than for Ca\textsuperscript{2+}. Mg\textsuperscript{2+} binds with an apparent dissociation constant of 2.5 mM, significantly weaker than either of the other two cations.

**Dimer Assembly Studies**

**Assembly Studies:** Initial studies test the ability of Mg\textsuperscript{2+} and Ni\textsuperscript{2+} to promote the formation of dimer as assessed by analytical SEC. In order to perform this study, we created an apo protein stock that had low intrinsic levels of the kinetically-trapped dimer. Data presented in Figure 2A
indicate that neither Mg\(^{2+}\) nor Ni\(^{2+}\) can promote the assembly of the D\(_{\text{sat}}\) dimer, while Ca\(^{2+}\) does. The level of dimer formed upon addition of 1 mM Ca\(^{2+}\) is consistent with the level of dimer expected based on the protein concentration and the K\(_d\) for dimerization.[39]

**Disassembly Studies:** To determine if Mg\(^{2+}\) or Ni\(^{2+}\) will disassemble D\(^*\)\(_{\text{apo}}\), the cations were added to an apo stock that was enriched in D\(^*\)\(_{\text{apo}}\). While 1 mM Mg\(^{2+}\) did promote some dimer disassembly, 1 mM Ni\(^{2+}\) had a greater effect. As expected, Ca\(^{2+}\) disassembled D\(^*\)\(_{\text{apo}}\) to create a dimer peak that was expected based on the final concentration of NCAD12 injected on the analytical SEC column (Figure 2B). The ability of Mg\(^{2+}\) and Ni\(^{2+}\) to disassemble D\(^*\)\(_{\text{apo}}\) suggests that these cations bind to the same binding sites as calcium.

**Figure 2:** (A) Assembly of dimer assayed by analytical SEC. A total concentration of Ca\(^{2+}\) (1 mM; ····), Mg\(^{2+}\) (1.5 mM; - - -), or Ni\(^{2+}\) (1 mM; ····) was added to an apo solution of NCAD12 that had a low level of kinetically-trapped dimer. Chromatograms illustrate that Ca\(^{2+}\) promotes the formation of D\(_{\text{sat}}\), while Mg\(^{2+}\) and Ni\(^{2+}\) do not. (B) Disassembly of kinetically trapped dimer assayed by analytical SEC. An apo NCAD12 stock was prepared with enriched levels of kinetically-trapped dimer (—). The apo stock (—) was disassembled with the addition of 1 mM Ca\(^{2+}\) (····), 1 mM Mg\(^{2+}\) (····) or 1 mM Ni\(^{2+}\) (·······). After addition of cation, the level of dimer decreased indicating that cation binding “unlocks” the trapped dimer that was in the apo stock.
Competition Experiments: To understand the linked equilibria between calcium binding and dimer formation in NCAD12, we performed analytical SEC studies to determine the impact of competition for calcium-binding sites by Ni\(^{2+}\) or Mg\(^{2+}\) on dimer assembly. These chromatographic experiments provided an indicator of the level of D\(_{\text{sat}}\) as a function of competing concentrations of Ni\(^{2+}\) or Mg\(^{2+}\) when the concentration of Ca\(^{2+}\) was at normal physiological levels (1 mM). As hypothesized, as the concentration of Ni\(^{2+}\) increased, we observed a reduction in dimerization affinity (increase in dissociation constant (K\(_d\))) from 26 \(\mu\)M in the absence of Ni\(^{2+}\) to 107 \(\mu\)M in the presence of 1 mM Ni\(^{2+}\) (**Figure 3A**). A smaller, but similar effect was observed for competition experiments with Mg\(^{2+}\) added as summarized in **Table 2**.

<table>
<thead>
<tr>
<th>[Ni(^{2+})]</th>
<th>K(_d)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>26 ± 7</td>
</tr>
<tr>
<td>100 (\mu)M</td>
<td>49 ± 6</td>
</tr>
<tr>
<td>500 (\mu)M</td>
<td>67 ± 5</td>
</tr>
<tr>
<td>1 mM</td>
<td>107 ± 8</td>
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</table>

<table>
<thead>
<tr>
<th>[Mg(^{2+})]</th>
<th>K(_d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>32 ± 8</td>
</tr>
<tr>
<td>1.5 mM</td>
<td>45 ± 5</td>
</tr>
</tbody>
</table>

**Table 2.** Assembly of the calcium-saturated dimer
Representative size exclusion chromatograms are shown in Figure 3B, and the height of the D*apo peak represents the level of calcium-saturated dimer in solution as a function of the concentration of protein and added Ni\textsuperscript{2+} or Mg\textsuperscript{2+}.

Figure 3: (A) Effect of Ni\textsuperscript{2+} on calcium-induced dimer formation assayed by analytical SEC. The concentration of protein was 15 µM with 1 mM Ca\textsuperscript{2+} only (—) or with 1 mM Ca\textsuperscript{2+} and 1 mM Ni\textsuperscript{2+} (- - -). Additional Ni\textsuperscript{2+} were tested as described in the Experimental Procedures. (B) Effect of Mg\textsuperscript{2+} on calcium-induced dimer formation assayed by analytical SEC. The concentration of protein was 25 µM with 1 mM Ca\textsuperscript{2+} only (—) or with 1 mM Ca\textsuperscript{2+} and 1 mM Mg\textsuperscript{2+} (- - -). Calculated values for the apparent dimer dissociation constants for each level of cation are shown in Table 1.
DISCUSSION

The studies reported here indicate that natural and non-physiological divalent cations in the extracellular microenvironment can modulate the dimerization affinity of N-cadherin. They do this by decreasing calcium-binding affinity due to competition for the binding sites. While neither of the two divalent metals we tested could directly induce formation of dimer, the observed decrease in calcium binding affinity due to binding site competition affects adhesive dimer formation.

Would we expect the calcium binding sites of cadherin to accommodate other divalent ions? The calcium-binding sites in cadherin are located at the interface between adjacent modular domains and comprise clusters of negatively charged residues. The involvement of multiple loops from individual domains and the peptide linker between domains implies greater adaptability to ionic radii and coordination geometries. While we have no direct evidence that Ni$^{2+}$ or Mg$^{2+}$ bind to the calcium-binding sites, the fact that Ni$^{2+}$ and Mg$^{2+}$ disassemble the kinetically-trapped dimer is consistent with their occupancy of the calcium-binding sites. There should be space for Ni$^{2+}$ or Mg$^{2+}$ in the calcium-binding sites given that Ca$^{2+}$ has an ionic radius of 0.99 Å, Ni$^{2+}$ has an ionic radius of 0.69 Å, and Mg$^{2+}$ has an ionic radius of 0.72 Å.[40]

Divalent nickel can adopt a variety of coordination geometries, including four, five and six ligand coordination spheres, while divalent magnesium firmly prefers a six ligand coordination sphere.[26] Notably, Ni$^{2+}$ has a propensity to exist in equilibrium between multiple coordination geometries, explainable by the ease of reversible ligand coordination at the axial position of square-planar complexes, while divalent magnesium exists in equilibrium between two hexa-coordinated isomeric states.[41] While the calcium-binding sites in NCAD12 might also be amenable to a variety of coordination geometries, explicit chelation geometry is likely
necessary to create strain in the monomer required to promote dimer formation.[38] Furthermore, studies by Rodriguez-Cruz et al.[42] established that water dissociates more readily from \( \text{Ca}^{2+} \) than from \( \text{Ni}^{2+} \) or \( \text{Mg}^{2+} \), suggesting that \( \text{Ni}^{2+} \) and \( \text{Mg}^{2+} \) may bind more weakly to potential chelation sites on NCAD12 due to the dehydration energy penalty. Calcium titration and dimer assembly data displayed in Figures 1 and 2 suggest that while \( \text{Ni}^{2+} \) and \( \text{Mg}^{2+} \) can occupy the calcium-binding sites, they are unable to cause the conformational change necessary to promote the formation of adhesive dimer.

The global effect of competition for binding sites on cell adhesion by cadherins was studied in pioneering cell-based studies by Prozialeck et al.[43] They demonstrated that elevated concentrations of divalent cations, particularly cadmium, can disrupt calcium-dependent intercellular junctions.[43] Continued discovery in this area resulted in the proposal of a model to account for potential extracellular and intracellular targets of metal toxicity.[44] In this model, Prozialeck and coworkers propose two modes for direct disruption of cadherin function by toxic metals: competition for occupancy of binding sites, resulting in a loss of adhesive function, and conformational change due to metal binding which changes the protein’s susceptibility to proteolysis suggesting a direct effect of cadmium on the extracellular domain. Pearson et al demonstrated that cellular exposure to 10 µM cadmium resulted in significant loss of E-Cadherin from cellular junctions, increased migration of \( \beta \)-catenin to the nuclear and perinuclear regions of the cell, and increased mRNA levels of protooncogenes \( c-jun \) and \( c-myc \), implicating potentially novel mechanisms for divalent-metal-induced carcinogenesis.[45] Thus, these studies indicate that changes in divalent ion concentrations can have both extracellular and intracellular effects upon cell adhesion.

Some known health effects nickel causes are skin allergies and lung fibrosis, along with
causing toxicity to the kidneys and cardiovascular system.[46, 47] Additionally, nickel exerts neurotoxic effects via the inhibition of acetylcholinesterase and Na⁺,K⁺-ATPase, as recently demonstrated in a rat model.[37] Nickel ions have also been shown to display differential effects on a variety of voltage-activated calcium channels, displaying binding affinities as high as several mM.[48] Because of this known disruption on calcium channels, it is logical to hypothesize that other divalent cations present in the extracellular matrix, such as Ni²⁺, could also disrupt the binding of Ca²⁺ to N-cadherin, thereby decreasing the formation of dimer, which is required for a panoply of physiological processes.

In order to provide a simple summary of the effect of divalent cations on calcium-induced dimerization by N-cadherin, we performed a simulation of the fraction dimer as a function of divalent cation concentration. Given that the dissociation constant for calcium is 50 µM[38] and for dimerization is 25 µM,[39] we simulated the formation of adhesive dimer as a function of extracellular calcium concentration (Figure 4, dashed line).

**Figure 4:** Simulation of the relationships between fraction dimer formed in titrations of NCAD12. The dashed line is the fraction dimer formed in a calcium titration of NCAD12. The solid line is the fraction of dimer formed in a Ni²⁺ titration of NCAD12 with a uniform concentration of calcium (1 mM) over the course of the titration. The protein concentration was 25 µM. The Kd for dimerization was 25 µM. The association constant for calcium was 20000/M and for Ni²⁺ was
The fraction dimer increases with an increase in calcium concentration such that by the concentrations of calcium expected in the extracellular space (1 to 3 mM), the level of dimer is 98% of its maximum level. Figure 4 also illustrates the decrease in calcium-induced dimer formation as the level of an alternative divalent cation increases, in this case nickel. When the concentration of nickel is equivalent to 200 µM, NCAD12’s $K_d$ for $\text{Ni}^{2+}$, there is only a 5% decrease in the simulated fraction of adhesive dimer in the presence of 1 mM calcium; the level of calcium is 20 times the $K_d$ for calcium and therefore calcium occupies the calcium binding sites leading to formation of dimer. After this point, as the cation concentration increases, it successfully competes with the calcium for the binding sites, effectively reducing the level of calcium-induced adhesive dimer. We find this representation of the data to be particularly useful as a practical guide to predict a physiological effect on the stability of adherens junctions in the case of abnormally high levels of divalent cations in the extracellular space.

The extracellular space in neural tissue is known to be a complex milieu. Monovalent and divalent cations are in high abundance relative to calcium, such that one might expect an effect on cell adhesion by calcium-dependent cell adhesion proteins. Recently published work from our laboratory illustrated this concept. Jungles et al showed that a decrease in pH or an increase in NaCl concentration promotes the formation of adhesive dimer by N-cadherin, indicating that dimerization is sensitive to the charge state of the protein and the level of monovalent ions in solution.[24] Calcium levels have been shown to flux in neural tissue based on a number of different factors.[49-51] Thus, the impact of the ionic character of the extracellular microenvironment is a relevant and complex topic in cadherin-mediated cell adhesion. Collectively, these experimental approaches parse contributions of a variety of
molecular species within the extracellular matrix on the stability and dynamics of adherens junctions.
CHAPTER II
IMPACT OF PH ON THE STRUCTURE AND FUNCTION OF NEURAL CADHERIN

INTRODUCTION

Neural (N-) cadherin is a transmembrane protein within adherens junctions that mediates cell-cell adhesion. It has 5 modular extracellular domains (EC1-EC5) that bind 3 calcium ions between each of the modules. Calcium binding is required for dimerization. N-cadherin is involved in diverse processes including tissue morphogenesis, excitatory synapse formation and dynamics, and metastasis of cancer. During neurotransmission and tumorigenesis, fluctuations in extracellular pH occur, causing tissue acidosis with associated physiological consequences. Studies reported here aim to determine the effect of pH on the dimerization properties of a truncated construct of N-cadherin containing EC1-EC2. Since N-cadherin is an anionic protein, we hypothesized that acidification of solution would cause an increase in stability of the apo protein, a decrease in the calcium-binding affinity and a concomitant decrease in the formation of adhesive dimer. The stability of the apo monomer was increased, and the calcium-binding affinity was decreased at reduced pH, consistent with our hypothesis. Surprisingly, analytical SEC studies showed an increase in calcium-induced dimerization as solution pH decreased from 7.4 to 5.0. Salt-dependent dimerization studies indicated that electrostatic repulsion attenuates dimerization affinity. These results point to a possible electrostatic mechanism for moderating dimerization affinity of the Type I cadherin family. Extrapolating these results to cell adhesion
in vivo leads to the assertion that decreased pH promotes adhesion by N-cadherin, thereby stabilizing synaptic junctions.

Extracellular pH in humans is actively maintained between pH 7.2 and 7.4, except in abnormal states such as metastatic cancer,[52, 53] diabetic ketoacidosis,[54] high levels of lactate,[55] critical illness,[56] and in normal and diseased brain tissue where the pH decreased to between 6.8[57, 58] and 6.2.[52] The effect of the microenvironment on cell-cell adhesion may play a significant physiological role in the stability of adherens junctions and the associated intracellular events. Our interest is in how decreased pH will affect adhesion by N-cadherin, a cell adhesion molecule in adherens junctions that is critical in neurological synapse formation.[3, 11] N-cadherin is the primary cell adhesion protein within synaptic adherens junctions at the transmission zone, and is directly exposed to proton flux during periods of increased synaptic activity.[59] N-cadherin expression is also up-regulated in tumor progression, angiogenesis, and metastasis of numerous types of cancer cells.[60-63] Tumors in active periods of growth have been shown to acidify due to the Warburg effect, a state that is consistent with tumor cell proliferation.[64, 65] Since N-cadherin is an anionic protein that requires calcium binding for proper function, pH may have a profound effect on its structure, stability, and function.

N-cadherin is a member of the classical or type I cadherin family consisting of five tandem repeating extracellular domains, a single-pass transmembrane region, and a conserved C-terminal cytoplasmic region. Formation of adhesive dimers between cadherins on apposing cell surfaces occurs through the formation of a strand-swapped structure, which forms via the exchange of the N-terminal βA-strand between juxtaposed EC1 domains. The strand-swapped structure is stabilized by docking of the side chain of a conserved tryptophan (W2) into the conserved hydrophobic pocket of the neighboring protomer (c.f. [66]). Since the strand-swapped
interface is located in the first domain of the EC region,[20, 67] the studies reported herein utilize the first two EC-domains of N-cadherin (NCAD12). The two-domain construct has been well characterized in Type I cadherins[18, 66, 68] and is the minimal functional unit required for calcium-dependent dimerization in vitro.

The interface between tandem EC domains contains amino acids that play critical roles in the adhesion process. Calcium-binding sites comprise clusters of negatively-charged carboxyl groups from both modular domains at the interdomain interface. The critical anionic residues in the NCAD12 calcium-binding pocket are E11, D67, E69, D103, D134, D136 and D194 in N-cadherin. Calcium-binding sites 1 and 2 are linked by the side chain oxygens of E11, E69, and D103. Sites 2 and 3 are linked by the side chain oxygens of D136, while site 3 comprises, in part, both side chain oxygens from D134.[68] Studies have shown that mutations of these residues resulted in a dramatic decrease in calcium-binding affinity and dimerization.[38, 69-71]

Isolated amino acid pKa values of acidic residues (4.5-3.3) are well outside the physiologically relevant range (7.4-6.0);[72] however, pKa values for acidic residues in folded proteins can differ by orders of magnitude from these canonical values, especially in acidic proteins where they have been shown to be as high as 9.[73-75] NCAD12 also contains three histidine residues (H75, H79, and H110) whose protonation state may change in a physiological relevant pH range. Histidine residues have been characterized as “pH sensors”,[76-78] whose protonation state can affect protein conformational stability in a physiological pH range.[79] Thus, we might expect significant protonation of critical acidic and histidine residues in a physiologically relevant pH range.

Since the residues comprising the calcium binding sites are highly anionic, we propose that a decrease in solution pH should stabilize NCAD12 due to the decrease in electrostatic
repulsion from neutralization of acidic residues. We also propose that an increase in solution acidity should introduce competition between calcium and protons for site occupancy, resulting in a reduction of calcium-binding affinity followed by a concomitant decrease in dimerization affinity. In this collective work published in 2015 (REF), we address the impact of pH on the stability, calcium-binding affinity, and the dimerization affinity of NCAD12 protomers using spectroscopic and chromatographic methods. In this thesis, I will highlight my experimental contribution to the work.
METHODS

Protein Expression and Purification

The cloning of the gene for the first two extracellular domains (residues 1-221) of NCAD12 (EC1, linker1, EC2, and linker2) was described previously.[39] Recombinant pET30 Xa/LIC plasmids were amplified by KOD HiFi DNA Polymerase (Stratagene) and transformed into *Escherichia coli* BL21 (DE3) cells.[39] Protein was overexpressed and purified as described in previous work.[38] Protein purity was verified via SDS-PAGE in 17% Tris-Glycine gels through standard protocols. The concentration of the protein stocks was determined spectrophotometrically ($\epsilon_{280} = 15,900 \pm 400 \text{ M}^{-1}\text{cm}^{-1}$).[80]

Dilution Method for pH Adjustment

In order to create protein solutions over a range of pHs (7.4-5.0), we prepared our stock at pH 7.4, but with a low buffer strength (2 mM HEPES). Identical diluent buffers at higher buffer strength were made at pHs 7.4, 7.0, and 6.5 (40 mM HEPES, 140 mM NaCl) and at pH 6.0, 5.5, and 5.0 (40 mM NaOAc, 140 mM NaCl). The NCAD12 stock in 2 mM HEPES at pH 7.4 was diluted 1 part protein stock plus 2.4 parts diluent buffer (e.g. 80 µM stock; 23.5 µM working concentration). We confirmed that this dilution ratio was sufficient to adjust the pH to the desired level by measuring pH with a microelectrode.

Thermal Unfolding Studies

Thermal unfolding studies as a function of calcium and pH (6.0, 6.5, 7.0, and 7.4) were performed on an AVIV 202SF Circular Dichroism (CD) Spectrometer. Solutions of 5 µM NCAD12 were placed in a 1 cm quartz cuvette with a fitted temperature probe inserted through the stopper. This concentration was chosen to minimize dimer formation while maximizing signal to noise. The solution was stirred throughout data acquisition. Data were acquired at 227
nm at a temperature range of 15-95 °C (1°C intervals with a 30 sec equilibration period and a 5 sec data averaging time). Thermal denaturation profiles were identical with expanded equilibration times (30 seconds to 2 minutes) during data acquisition indicating that the kinetics of unfolding were rapid (Supporting Information). The calcium-saturated samples were brought to 2.5 mM total calcium concentration to ensure maximum saturation of binding sites at all pH values. Data were fit to the Gibbs-Helmholtz equation with linear native and denatured baselines as described previously.[81] \( T_m \) and \( \Delta H_m \) values were allowed to vary in fits to this equation, while \( \Delta C_p \) was fixed to 1 kcal mol\(^{-1}\) K\(^{-1}\).[39] \( \Delta C_p \) was also fixed to 0 kcal mol\(^{-1}\) K\(^{-1}\) and 2 kcal mol\(^{-1}\) K\(^{-1}\) to determine the effect of its value on resolved values of \( \Delta H_m \) and \( T_m \). Variation, as a function of the value for \( \Delta C_p \), was smaller than the standard deviation in the resolved parameters. Thermal denaturation is a reversible process in NCAD12, however, refolding is slower than unfolding likely due to the abundance of prolines in the protein.[82]

**Calcium Titrations**

The CD signal of NCAD12 was monitored during calcium titrations using an Olis DSM 20 CD Spectrometer. Solutions of 1, 10, 100, and 700 mM CaCl\(_2\) were each added sequentially in 2.5, 5.0, and 10.0 µL increments to 5 µM NCAD12 in solutions with pH values between 7.4-5.5. Samples were stirred throughout the titration. Titrations were performed at least twice. CD signal was recorded in wavelength scans from 300-210 nm with an averaging time (1-13 seconds) that was proportional to dynode voltage at each wavelength. To resolve free energy changes for calcium binding, titration data were considered at wavelengths from 230-220 nm to optimize the signal to noise ratio. Titration data were fit to an equation for equal and independent sites with linear apo and saturated baselines. While we expected cooperative
binding of calcium, data did not support analysis by a more complex model based on the span and randomness of residuals of fitted data.

In order to determine the apparent pK\textsubscript{a} for the protonation event(s) that affected calcium binding affinity, we used a simple competitive model as described by the equations below

\[
\frac{K_X \cdot X \cdot M_o \cdot (Z)}{1 + K_X \cdot X \cdot (Z)} = MX
\]

\[
Z = \left(1 - \frac{K_Y \cdot Y}{1 + K_Y \cdot Y}\right)
\]

where \(M_o\) is the total protein concentration, \(X\) is the concentration of calcium and \(Y\) is the concentration of protons. \(K_x\) is the association constant for calcium and \(K_y\) is the association constant for protons. Prior to analysis data were normalized to endpoints from individual fits, and then endpoints were fixed in the global analysis to determine the values of \(K_x\) and \(K_y\).
RESULTS

Thermal Unfolding Studies

Thermal unfolding studies were performed to observe the impact of pH and calcium on the stability of NCAD12. The CD signal was monitored as a function of temperature. Two distinct transitions were observed with the signal becoming increasingly negative as the protein unfolded, consistent with a polyproline conformation of the unfolded state.[83] Previous work has shown that the first transition corresponds to unfolding of EC2 and the second transition to unfolding of EC1.[84] In the absence of calcium, NCAD12 was soluble and monomeric (M_{apo}) from pH 7.4 to 6.0. NCAD12 precipitated during denaturation experiments at pH values approaching its pI, so we were unable to obtain estimates of stability at pH values below 6.0 in the apo state and below 6.5 in the calcium-saturated state (M_{sat}). In the apo-state, the apparent stability of the protein increased as pH decreased (Figure 5). Data for the first transition were fit to the Gibbs-Helmholtz equation with adjustable baseline parameters. The values resolved for T_m increased significantly as the pH decreased while the values resolved for ΔH_m decreased slightly leading to a net increase in calculated values for ΔG^* at 37 °C (Table 3).

<table>
<thead>
<tr>
<th>Buffer</th>
<th>ΔH_m (kcal/mol)</th>
<th>T_m (°C)</th>
<th>ΔG^{ab} (kcal/mol)</th>
<th>ΔH_m (kcal/mol)</th>
<th>T_m (°C)</th>
<th>ΔG^{ab} (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.4</td>
<td>74 ± 3</td>
<td>44.0 ± 0.1</td>
<td>1.6 ± 0.2</td>
<td>90 ± 3</td>
<td>56.6 ± 0.1</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>71 ± 3</td>
<td>44.7 ± 0.2</td>
<td>1.6 ± 0.1</td>
<td>91 ± 5</td>
<td>56.3 ± 0.2</td>
<td>4.8 ± 0.1</td>
</tr>
<tr>
<td>pH 6.5</td>
<td>71 ± 3</td>
<td>46.0 ± 0.2</td>
<td>1.9 ± 0.2</td>
<td>94 ± 2</td>
<td>56.2 ± 0.1</td>
<td>4.9 ± 0.2</td>
</tr>
<tr>
<td>pH 6.0</td>
<td>67 ± 2</td>
<td>48.5 ± 0.5</td>
<td>2.2 ± 0.2</td>
<td>N/A^c</td>
<td>N/A^c</td>
<td>N/A^c</td>
</tr>
</tbody>
</table>

a Gibbs Helmholtz equation where ΔC_p was fixed at 1 kcal mol^{-1} K^{-1}[39]. Reported errors were resolved from global analysis of replicate experiments.
b Values were calculated at 37 °C.
c Values were not resolved due to protein precipitation.

Table 3. Results from Thermal Denaturation Experiments
In the presence of calcium, the thermal-denaturation profiles were indistinguishable over the accessible pH range. Identical values resolved for $T_m$, $\Delta H_m$, and calculated values for $\Delta G^\circ$ at 37 °C indicate that the binding of calcium masked the effect of decreased pH that was observed in the apo state. Analysis of the second transition (unfolding of EC1) was problematic due to protein precipitation at pH 7.0 or less at temperatures at which the EC1 transition was occurring.

**Ca**lci**um Titrations**

The pH dependence of calcium-binding affinity of NCAD12 was assessed via calcium titrations monitored by CD spectroscopy. Figure 6A shows the CD signal as a function of calcium concentration at each pH. The data at each pH were normalized to correct for the pH dependence of the magnitude of the CD signal, and then offset for clarity. The CD signal increased (less negative) with the addition of calcium at all pH values consistent with a calcium-induced changes in structure. Since the span of the CD signal over the course of titrations performed at pH 6.0 and 5.5 was low ($\Delta \Delta \varepsilon = 2.3 \pm 0.3$ mdeg), the signal to noise ratio was poor.
Studies were also attempted at pH 5.0; however, protein precipitated during the titration. NCAD12 was >85% saturated at 1 mM Ca$^{2+}$ at all pH values.

Based on the randomness and span of residuals, data fitted well to a binding model of equal and independent sites indicating that there was no observed cooperativity in calcium binding from pH 7.4 to 5.5. There was a small, yet systematic, decrease in calcium-binding affinity as pH decreased from 7.4 to 6.0 with a significant decrease in affinity as the pH was decreased further to 5.5. This pH-dependent difference is reflected in resolved values for $K_a$ in Table 4 ($K_{a7.4} = 3 \cdot K_{a6.0}$ and $K_{a6.0} = 3 \cdot K_{a5.5}$).

Proton binding decreases the apparent calcium binding constant (Table 4). In order to estimate the pK$_a$ of the relevant protonation event(s), calcium binding data as a function of pH were fit to a competitive binding model given in Eqs 1 and 2. Data at all pH values were

**Figure 6:** Calcium titrations of NCAD12 as a function of pH. NCAD12 was titrated with calcium at pH 7.4 (●), 7.0 (○), 6.5 (▼), 6.0 (∆), and 5.5 (■); the normalized CD signal is plotted against total calcium concentration. (A) Representative titrations at each pH are shown. Solid lines are simulated based of parameters resolved from global analysis of at least two separate experiments. Data are normalized and offset for clarity. Midpoints of transitions are designated by X. (B) To resolve the pK$_a$ of the protonation event that impacted the apparent calcium binding affinity at each pH, data were analyzed according to a competitive binding model. Resolved values for the changes in free energy of binding are shown in Table 4.
Simultaneously analyzed to determine the binding constants for calcium and protons. Resolved values are in **Table 4**.

<table>
<thead>
<tr>
<th>pH</th>
<th>$K_a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>$35 \pm 10 \times 10^3$</td>
</tr>
<tr>
<td>7.0</td>
<td>$25 \pm 8 \times 10^3$</td>
</tr>
<tr>
<td>6.5</td>
<td>$18 \pm 7 \times 10^3$</td>
</tr>
<tr>
<td>6.0</td>
<td>$13 \pm 6 \times 10^3$</td>
</tr>
<tr>
<td>5.5</td>
<td>$4.7 \pm 1.4 \times 10^3$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Competitive Model$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_a(Ca^{2+})$</td>
</tr>
<tr>
<td>$K_a(H^+)$</td>
</tr>
<tr>
<td>pKa$^c$</td>
</tr>
</tbody>
</table>

$^a$ Apparent association constants for calcium from analysis of data at each pH independently.

$^b$ Fits of data to Eq 1 and 2 yielding apparent association constants for calcium ions and protons.

$^c$ pKa value is the log of the proton association constant.

**Table 4. Free Energies of Calcium Binding Resolved from Analysis of Calcium Titrations**

The $K_a$ for calcium from individual analysis of data at pH 7.4 was similar to that found from analysis of calcium binding data at pH 7.4, the reference pH for the experiments presented here. The apparent pKa (log of the association constant for protons) for the proton binding event(s) that perturbs calcium binding is $6.0 \pm 0.1$, a value close to the canonical value for histidine or an acidic residue such as aspartate or glutamate.
DISCUSSION

N-cadherin, a member of the classical cadherin family, is an acidic protein that requires calcium for its function as a primary cell-cell adhesion molecule in adherens junctions. It plays pivotal roles in tissue development,[85, 86] cancer[60, 63, 87] and neurological synapses.[6, 88] Due to its prominent physiological role, the structure-function relationship of N-cadherin has been of interest recently. In particular, our laboratory is interested in the effect of microenvironment on the adhesive properties of N-cadherin. Given the fact that key physiological processes cause acidification of tissue, studies reported herein assess the effect of pH on N-cadherin function. Since we expected that protonation of calcium-binding residues would decrease calcium-binding affinity, and calcium binding is required for dimerization, then it follows that dimerization affinity should also decrease.

We observed an increase in stabilization of NCAD12 as pH decreased. Thermodynamic parameters in Table 3 provide insight into the increased stabilization of EC2 as a function of pH. Based on earlier studies of isolated EC2 of E-[89] and N-cadherin,[90] we expected to see an increase in T_m and a concomitant increase in the enthalpy change (ΔΔH_m) at T_m, as net charge was reduced. However, while T_m increased in these pH-dependent studies, enthalpy decreased, albeit slightly (ΔΔH_m of 7 kcal/mol). Bechtel et al state that enthalpy of protonation is not a function of pH in studies of protein stability.[91] The linear relationship between ΔH_m and T_m allows prediction of ΔC_p, yielding a ΔC_p for protonation close to 0 kcal/ (mol K). If we consider the increase in T_m, assuming ΔH_m to be constant, it would imply some decrease in entropy at T_m (T_m*ΔS_m = ΔH_m; when ΔH_m is constant). This result indicates that decreasing the pH produces a decrease in conformational entropy, an increased exposure of hydrophobic surface area, or a combination of both.[92] Enthalpy changes for single protonation events of aspartates and
glutamates are relatively small compared to those for protein folding ($\Delta H_p$ of 0.5 to 1.5 kcal/mol),[93] possibly leading to the small, observed change in the enthalpy of unfolding as a function of pH.

We hypothesized that protonation of acidic residues in the calcium-binding pocket should lead to an overall decrease in the calcium-binding affinity. As predicted, we found that a decrease in pH did decrease calcium-binding affinity; higher calcium levels are required to populate calcium-binding sites at lower pH values. However, the magnitude of this decrease was only a factor of three over a 1.5 pH unit range (7.4-6.0). Additional calcium titrations at pH 5.5 showed the same reduction of a factor of three over a smaller pH unit range (6.0-5.5), indicating that the protonation of key acidic residues decreases the calcium binding affinity.

Comparing the parameters resolved from the competitive binding model to the predicted value for pKa of calcium-binding residues (data not shown) indicates that D134 may be the key player in the pH-dependence of calcium binding. This residue in particular is the key requirement for calcium occupancy of the calcium-binding sites.[38, 71, 94] Our data indicate that when the pH drops below the predicted pKa for D134, there is a profound impact in the apparent calcium binding constant. Histidine 79 is adjacent to A80 which is in the hydrophobic pocket. Thus, it is also possible that protonation of this residue changes the conformation of the hydrophobic pocket and influences the calcium binding affinity.[95, 96]

In conclusion, within the pH range (7.4 to 5.0) NCAD12 showed a pH-dependent decrease in calcium-binding affinity and increase in dimerization affinity. Since the pH in metastatic cancers is depressed by up to 1.2 pH units[52, 53] as is the pH at neurological synapses,[52, 57] we would predict small increases in N-cadherin dimerization affinity. The extracellular neurological microenvironment is complex and dynamic.[97] These
microenvironmental factors include local fluxes in calcium concentration\cite{51} and other metals\cite{98} that could compete with the calcium and have a profound affect dimerization by cadherins.

In terms of the structure-function relationship of the classical cadherin family, the change in dimerization affinity is consistent with electrostatic repulsion playing a role in moderating the affinity of dimerization. This observation begs the question of whether electrostatic interactions are definitive contributors to the tuning of the relative affinities of N-, E- and P-cadherin at the strand-crossover or X-dimer interfaces, thereby playing a role in the equilibria and kinetics of adhesion by classical cadherins.
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VITA

MATTHEW PAUL DUKES

321 LeBourgeois Lane • Brandon, MS 38655 • (662) 915-5328 • mpdukes1@gmail.com

EDUCATION

M.S., Chemistry, University of Mississippi, May 2017

Thesis: Nickel Reduces Calcium Dependent Dimerization in Neural Cadherin

B.A. (Honors), Biochemistry, University of Mississippi, May 2009