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Calcium-Induced Strain in the Monomer Promotes Dimerization in Neural Cadherin

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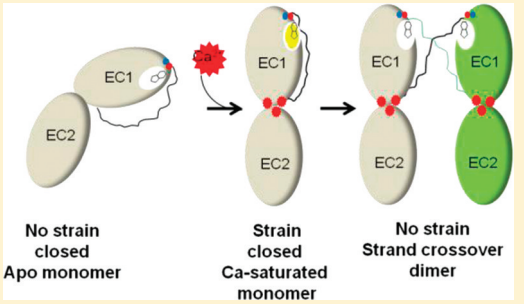
ABSTRACT: Cadherins are cell adhesion proteins that are important for tissue formation and integrity. Cell−cell adhesion occurs through the formation of the strand-crossover dimer between identical cadherins on the surface of neighboring cells. The strand-crossover dimer forms exclusively between their EC1 domains via swapping of the βA sheet by undocking the conserved tryptophan 2, W2, from its own hydrophobic pocket and docking it into the hydrophobic pocket of its adhesive partner. An interesting aspect of the system is the fact that critical noncovalent interactions in the monomer re-form in the dimer. Thus, as these noncovalent interactions are conserved, what drives the formation of dimer? Moreover, why is dimer formation calcium-dependent? Thus, to probe the structural and energetic effects of calcium on the noncovalent interactions that are necessary for dimer formation, we performed spectroscopic, stability, and assembly studies of wild-type and two mutants, W2A and E89A, of neural (N-) cadherin. We find that while the ionic interaction involving E89 has a minimal effect on the general stability of the closed conformation of the βA sheet, the hydrophobic interaction involving W2 is the source of the calcium requirement for adhesive dimer formation. The binding of calcium creates strain in the W2−hydrophbic pocket interaction through direct connection of E11 at the C-terminus of the βA sheet to calcium. To overcome this unfavorable condition in the monomer, N-cadherin forms a dimer. Taken together, our data provide a thermodynamic basis for the calcium dependence of strand-crossover dimer formation in N-cadherin.



The cadherin family comprises cell adhesion molecules that are important for tissue formation and integrity. Classical cadherins have an extracellular region with five modular domains numbered EC1−EC5 that mediate calcium-dependent cell−cell adhesion. Cell−cell adhesion occurs through the formation of a strand-crossover dimer[1−3] between identical cadherins on the surface of neighboring cells. The strand-crossover dimer forms exclusively between their N-terminal extracellular modules (EC1) and requires particular symmetrical noncovalent interactions at the dimer interface. Although calcium is required for dimerization in vivo, it is not clear what calcium binding actually does to the molecule to facilitate strand-crossover structure formation. Here, we report biophysical studies of the first two ectodomains of neural cadherin (N-cadherin) that address the thermodynamic role of the critical noncovalent interactions in the formation of the strand-crossover dimer and the role of calcium in this process.

An adhesive dimer forms between EC1 domains through swapping of βA sheets to form the strand-crossover structure. A model for the formation of the strand-crossover structure and the critical noncovalent forces that stabilize this structure is illustrated in Figure 1A. Hydrophobic and ionic interactions are the critical components of this model.[2,4] First, in the absence of calcium, N-cadherin forms a closed monomer in which a tryptophan in the second position, W2, is docked into a hydrophobic pocket of its own subunit, and there is a favorable ionic interaction between the N-terminus and the side chain of a glutamate residue, E89. Second, the binding of calcium induces the formation of a strand-crossover structure in which the ionic and hydrophobic interactions re-form symmetrically across the strand-crossover dimer interface. It is important to note that while the N-terminal end of the βA sheet participates in these noncovalent interactions for the formation of the strand-crossover dimer, the C-terminal end of the βA sheet has an essential calcium binding amino acid, E11, that is the only direct link to calcium binding (cf. ref 1). Thus, the disposition of the βA sheet, and its noncovalent interactions, is directly linked to calcium binding (Figure 1B). At this point, there have been a number of different structural[1,2,5,6] and functional[7−10] studies that support the strand-crossover structure as the adhesive dimer interface.

While the end states of dimerization in cadherin, apo monomer and calcium-saturated strand-crossover dimer, have been well characterized, the exact nature of the intermediate state is an area of active research. To understand the basic mechanism of dimerization, one must know the pathway between the end states. One scenario is that calcium binding induces opening of the βA strand, thereby exposing the N-terminus and W2 (Figure 1A). Two of these open structures then form a strand-crossover dimer in which the ionic interaction is re-formed and W2 is docked in the partner subunit. This scenario was supported by studies on a series of mutants of N-cadherin by Harrison et al. that indicated that the binding of calcium increases exposure of W2 and changes the

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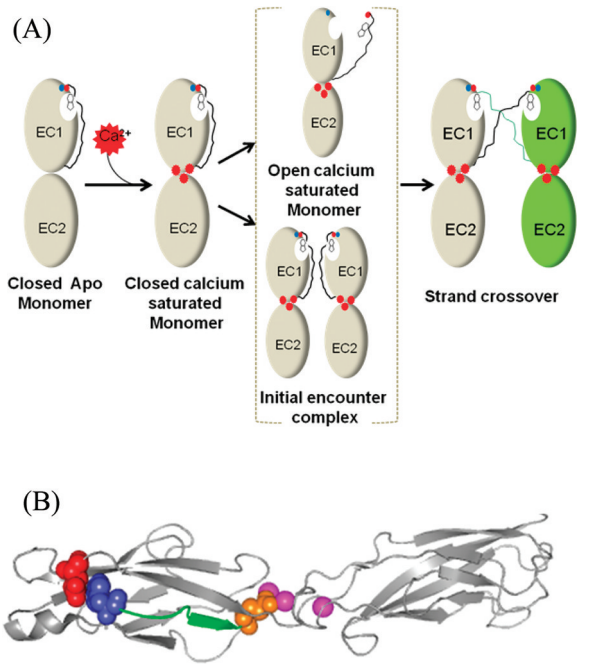

Figure 1. (A) Schematic of calcium-induced formation of the strand-crossover dimer. NCAD12 is illustrated as EC1 and EC2 modules. The noncovalent interactions in the closed structure are illustrated: the hydrophobic interaction between the docked W2 and the hydrophobic pocket in EC1 and the ionic interaction between the N-terminus (blue circle) and the side chain of E89 (red circle). In the apo state, the monomer is closed. When calcium binds, the closed calcium-saturated monomer is formed. The intermediate state between the closed calcium-saturated monomer and the closed strand-crossover dimer is unknown. The green bubble diagram represents the neighboring cadherin. (B) Ribbon representation of the first two domains of E-cadherin (PDB entry 1FF5[8]). In the ribbon structure, the βA sheet (green), tryptophan 2 (blue), glutamate 89 (red), glutamate 11 (orange), and calcium ions (magenta) are shown.

conformation in the hydrophobic pocket.[11] Another scenario for the nature of the intermediate state in the formation of the strand-crossover dimer is that an initial-encounter complex forms between protomers. Evidence for this initial-encounter complex was illustrated by Sivasankar et al. in single-molecule fluorescence resonance energy transfer and force measurement experiments.[12] This initial-encounter complex may be related to the X-dimer structure[13] and may also be important for the dissociation of the strand-crossover dimer.[14] Thus, this topic is being actively considered by several research groups.

An interesting aspect of this system is the fact that the critical noncovalent interactions are the same in the closed monomer and in the closed dimer. Thus, as there is no net change in the noncovalent interactions, what drives the formation of the dimer? Because dimer formation requires calcium, our focus is on the effect of calcium on the particular hydrophobic and ionic interactions that are important for stabilization of the closed structure. In the studies reported here, we study the properties of the closed monomer species that is calcium-saturated. This species holds the key to understanding the energetic linkage between calcium binding and dimer formation.

We present studies of a construct that contains the first two ectodomains of N-cadherin, NCAD12. This construct is ideal for our purposes for several reasons. First, it is the minimal functional construct that has an intact calcium-binding pocket. Second, it forms strand-crossover dimer whose dissociation rate depends on calcium concentration.[15] Third, it has a tryptophan in each of the two EC domains: W2, the critical tryptophan in EC1 at the strand-crossover interface, and W113 in EC2. These tryptophans are useful as reporter groups for calcium-dependent conformational changes in their respective domains. Thus, to probe the structural and energetic effects of calcium on the noncovalent interactions that are necessary for dimer formation, we report studies of wild-type NCAD12 and two tryptophan mutants, W2A to observe the signal of W113 alone and W113F to observe the signal of W2 alone. In addition, the role of E89 is explored through studies of the E89A mutant of NCAD12. Calcium-dependent fluorescence emission, thermal stability, and assembly studies were performed to monitor the impact of these mutations on the structure, stability, and dimerization of N-cadherin. These studies provide a thermodynamic argument that the binding of calcium induces strain in the W2–hydrophobic pocket interaction in the closed monomer that is relieved upon formation of the strand-crossover dimer.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. The construction of the first two ectodomains (residues 1–221), designated as NCAD12 (EC1, linker 1, EC2, and linker 2), was described previously.[15] Point mutations were introduced into the NCAD12 sequence by site-directed mutagenesis by using the Quickchange kit (Stratagene) with the following sense primers: W2A Sense, 5′ GGT ATT GAG GGT CGC GAC GCG GTC ATC CCG CC 3′; W113F Sense, 5′ CAC CAG GTT TTC AAT GGG TCT GTT CCA GAG GGA 3′; E89A Sense, 5′ G GAC ATC AAT GGC AAT CAA GTG GCG AAC CCC ATT GAC ATT GAC 3′. Amplification of the two-domain constructs, digestion of template DNA, ligation into pET30 Xa/LIC, and subsequent transformation into *Escherichia coli* BL21(DE3) were performed according to standard protocols, utilizing KOD HiFi DNA Polymerase (Stratagene) and the Xa/LIC cloning kit (Novagen). Point mutations were confirmed by plasmid sequencing.

Overexpression and Purification. Overexpression and purification of the protein were described previously.[15] Digested NCAD12-WT (wild-type), NCAD12-W2A (W2A), NCAD12-W113F (W113F), and NCAD12-E89A (E89A) proteins were further purified using size exclusion chromatography (SEC) with a Sephacyl S-100 (Amersham) 1.2 cm × 0.5 m (~100 mL) column in 140 mM NaCl and 10 mM HEPES (pH 7.4). The concentration of residual calcium in the SEC buffer was found to be less than 1 μM by inductively coupled plasma optical emission spectroscopy. The purity of proteins was assessed by SDS–PAGE in 17% polyacrylamide gels. Extinction coefficients were determined experimentally on the basis of the amino acid composition using a molar absorption coefficient of tryptophan residues and tyrosine residues in the denatured state.[16] The extinction coefficients at 280 nm were 17700 ± 500 M^{-1} cm^{-1} for the wild type, 11800 ± 400 M^{-1} cm^{-1} for W2A, 17300 ± 400 M^{-1} cm^{-1} for E89A, and 11650 ± 350 M^{-1} cm^{-1} for W113F.

Determination of the Surface Accessibility of W2 and W113. N-Cadherin crystal structures 1ncj[7] and 1ff5[8] were used to determine the surface accessibility of the side chains of W2 and W113 by using GetArea.[17] Surface accessibility estimates of W2 and W113 showed that 10% of W2 and 40% of W113 are accessible to water. Thus, W113 is in a

significantly more polar environment than W2 in the closed structures.

Calcium Binding Studies. Calcium titrations were monitored by the change in the circular dichroism signal using an AVIV 202SF circular dichroism (CD) spectrometer at 225 nm. The protein solution (2.5 μM) in 10 mM HEPES and 140 mM NaCl (pH 7.4) was placed in a 1 cm path cuvette at 25 °C while the solution was stirred and titrated with calcium by addition of small volumes of calcium chloride stocks at different concentrations (0.1, 1, 10, and 100 mM). The total calcium concentration was assumed to be equivalent to the free calcium concentration. The spectral signal of the sample before calcium was added was assigned a free calcium concentration of 1 μM.[15] The calcium titration data were fitted to a model of equal and independent binding shown in eq 1

$$\bar{Y} = \frac{k_a X}{1 + K_a X} \quad (1)$$

where $\bar{Y}$ is the fractional saturation of sites and K_a is the calcium association constant. This experiment yielded estimates of the midpoint of the calcium binding titrations, but not the stoichiometry. There was no evidence of cooperativity in the binding of calcium.

Thermal Unfolding Studies. Thermal unfolding studies employed an AVIV 202SF circular dichroism (CD) spectrometer in the absence and presence of calcium (1 mM). The temperature ramp rate was 1 °C/min, and the temperature range was from 15 to 90 °C. The Gibbs–Helmholz equation was used to analyze the data as described previously.[18]

Native Gel Electrophoresis. Native gel electrophoresis was used to confirm the size of the protein samples. Under the native condition, the wild type and mutants have the same pI (4.35). The charge of E89A is reduced because of the mutation from −9.3 to −8.3.[19] Given this similarity, they should separate on the basis of their size. The molecular masses of these proteins in the monomeric state are 24 kDa and in dimeric state 48 kDa. To analyze the purified protein samples, 10 μL of a 45 μM protein solution was mixed with 10 μL of native loading buffer (pH 6.8), and 10 μL of this mixture was loaded on the 17% PAGE gel. Human serum albumin was used as a standard for the size of the dimer because it has a pI and Stokes radius similar to those of the dimer.[20] Electrophoresis was performed at 150 V for 1 h at 4 °C, and then the gel was stained with Coomassie blue.

Assembly Studies. Analytical size exclusion chromatography (SEC) was used to indicate the presence of dimer in protein stocks. SEC was performed with a Superose-12 10/300 GL column (Amersham) using an ÄKTA Purifier high-performance liquid chromatography system with UV absorbance detection at 280 nm. The mobile phase consisted of 140 mM NaCl and 10 mM HEPES (pH 7.4) with a flow rate of 0.5 mL/min. The injection volume was 50 μL, and the protein concentration was 50 μM. The calibration of the SEC column was described previously.[20] We observed the level of monomer and dimer as the height of the peaks detected at 280 nm. To observe the effect of calcium on dimerization, in another set of experiments, 1 mM calcium was added to the protein stocks and to the mobile phase. Protein stocks were assayed by analytical SEC as a function of protein concentration. Dimerization was assessed as a change in elution volume.

Spectral Studies. Fluorescence emission (FL) spectral scans were performed on a PTI QuantaMaster spectrofluorometer with excitation at 295 nm and emission scanned from 300 to 425 nm with a 1 s averaging time at 25 °C. The proteins were in 10 mM HEPES and 140 mM NaCl (pH 7.4) (apo) with 1 mM calcium added. The protein concentration was 5 μM. To monitor the complete exposure of W2 and W113, we denatured the proteins in 8 M guanidine hydrochloride (GHCl) and recorded the FL emission spectra.

RESULTS

Calcium Binding Studies. The calcium-dependent changes in the CD signal for the wild type, E89A, W2A, and W113F were monitored during calcium titrations. Representative calcium titrations are shown in Figure 2 with resolved free energies of binding reported in Table 1. The CD signal for all four proteins increased as calcium was added. They had similar affinities with best-fit values of the free energy change differing by only 0.2 kcal/mol, indicating that the mutations do not have a significant impact on the calcium binding affinity.

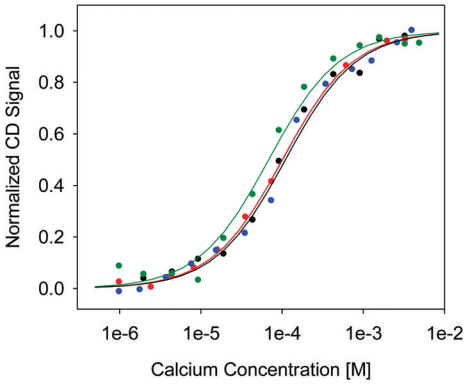

Figure 2. Calcium titrations of the wild type and mutants. The CD signal was monitored for each protein at its maximum wavelength and plotted vs the total calcium concentration for the wild type (black), W113F (green), W2A (blue), and E89A (red). The lines are simulated on the basis of parameters resolved from fits to eq 1. Resolved free energies are listed in Table 1.

Table 1. Summary of Free Energy Changes Resolved from Fits to the Adair Equation[a]

protein	$\Delta G°$ (kcal/mol)
wild type	−5.4 ± 0.3
W2A	−5.6 ± 0.3
W113F	−5.4 ± 0.4
E89A	−5.4 ± 0.2

[a]Reported errors are either the standard deviation in the average of the best fit value for the parameter or the largest error in the resolved parameter on an individual fit, whichever is larger.

Thermal Unfolding Studies. Thermal unfolding studies were performed to monitor the impact of the mutations on the structure and stability of the proteins. The CD signal at 230 nm from thermal denaturation experiments was plotted as a function of temperature in the absence (Figure 3A) and presence of calcium [1 mM (Figure 3B)]. Under both solution conditions, two transitions were observed for the wild type and W113F. The first- and second-transition data for the wild type and W113F (Figure 3A) are in agreement with unfolding of the

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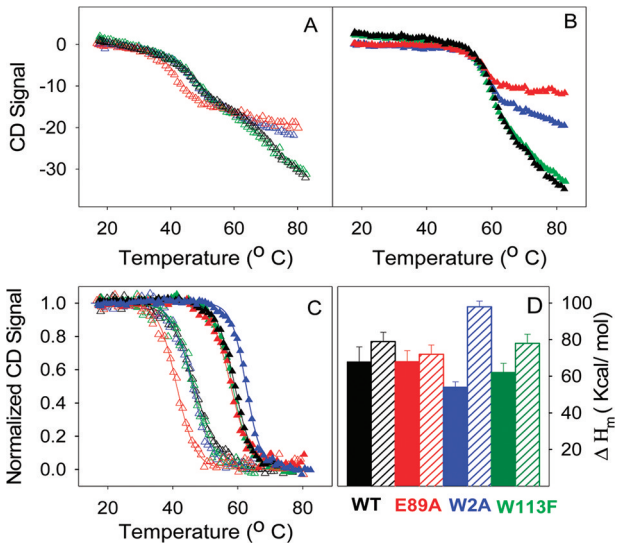

Figure 3. Thermal unfolding of the wild type and mutants. The raw CD signal vs probe temperature is represented for the wild type (black), W113F (green), W2A (blue), and E89A (red) in (A) the apo state and (B) the calcium-saturated state. (C) Data from the first transition were fit to the Gibbs−Helmholtz equation, normalized on the basis of the fitted native and unfolded baselines, and plotted vs temperature. Lines are simulated on the basis of resolved parameters. (D) To highlight the difference in the resolved values for the enthalpy change of unfolding, the enthalpy change (kilocalories per mole) is plotted for the apo (solid bars) and calcium-saturated (cross-hatched bars) states. Error bars represent the standard deviation in the best fit value.

isolated domains EC2 (T_m = 54 ± 1 °C[21]) and EC1 (T_m = 70 ± 4 °C[22]). Thermal unfolding studies with W2A and E89A showed only one transition. We are convinced that this single transition corresponds to the first transition (EC2) because of the position and the span of the data. Both proteins lost the second transition that corresponds to the unfolding of EC1, the domain in which the mutations occur. Because the hydrophobic interactions of W2 and the ionic interactions of E89 both pertain to the structural disposition of the βA sheet in EC1, this loss of CD signal from EC1 may indicate that the βA sheet is the primary contributor to the CD signal from EC1. Because W2A and E89A both bind calcium, we believe that EC1 is folded. Regardless, there is a manifestation of the W2A and E89A mutations in EC1 on the stability of EC2. The first- and second-transition data were analyzed separately to provide estimates for the enthalpy and temperature of the unfolding transition.

First Transition. The data from the first transition were fit to a two-state model according to the Gibbs−Helmholtz equation providing numerical estimates for T_m and ΔH_m. The baseline-corrected results from the analysis of the first transition are plotted in Figure 3C. The solid lines are simulated data based on the best fit parameters as reported in Table 2 along with calculated values for $\Delta G°$ at 25 °C. The resolved values of ΔH_m are also illustrated in the bar chart in Figure 3D to highlight the calcium-dependent differences in this parameter because enthalpy makes the largest contributor to the free energy difference between these proteins. In the apo state, the wild type, E89A, and W113F had similar ΔH_m values. In comparison, W2A had a lower ΔH_m value. To assess whether the differences in resolved values of enthalpy were significantly different from that of the wild type, we used the Student's *t* test

Table 2. Summary of Parameters Resolved from the First Transition of the Thermal Unfolding Experiments for the Wild Type and Mutants in the Absence and Presence of Calcium

protein	buffer	ΔH_m[a] (kcal/mol)	T_m (°C)	$\Delta G°$ at 25 °C (kcal/mol)
wild type	apo	68 ± 8	45 ± 2	3.6 ± 0.5
	Ca^{2+}	79 ± 5	59 ± 3	6.3 ± 0.7
W2A	apo	54 ± 3	45 ± 1	2.8 ± 0.2
	Ca^{2+}	98 ± 5	61 ± 1	8.5 ± 0.2
W113F	apo	62 ± 5	46 ± 1	3.4 ± 0.3
	Ca^{2+}	78 ± 3	58 ± 1	6.1 ± 0.2
E89A	apo	68 ± 6	41 ± 1	3.1 ± 0.3
	Ca^{2+}	72 ± 5	57 ± 1	5.5 ± 0.1

to compare the values from the mutants to that of the wild type.[23] We found that while W113F and E89A were not different from the wild type in the apo state, W2A was (80% probability). Interestingly, the W2A mutation in EC1 impacts the stability of EC2, which indicates that there is active communication between domains even in the apo state.

In the calcium-bound state, the T_m values for all four proteins varied from 57 to 61 °C. These T_m values are relatively similar in comparison to the resolved ΔH_m values, which varied from 72 to 98 kcal/mol (Table 2 and Figure 3D). On the basis of the Student's *t* test, resolved values of ΔH_m for W113F and E89A were not different from that of the wild type, but W2A was different from the wild type in the calcium-saturated state (92% probability). Calculated values for $\Delta G°$ in the calcium-bound state for the wild type (6.3 kcal/mol), W113F (6.1 kcal/mol), and W2A (8.7 kcal/mol) were greater than that for E89A (5.5 kcal/mol). These results indicate that the E89A mutation decreased the stability of the protein, which implies that E89 in the wild type stabilizes the protein. In contrast, W2A is significantly more stable than the wild type, which implies that there is a free energy penalty in the wild type because of the interactions of W2 with the hydrophobic pocket.

Second Transition. The important feature of the second transition is that its position is the same for W113F and the wild type in the absence and presence of calcium. These results suggest that the W113F mutation did not affect the structure or stability of EC1. A more important observation is that because calcium does not impact the stability of the second transition it must dissociate before the EC1 domain unfolds. The melting temperatures for the second transition were estimated because they could not be fit to the Gibbs−Helmholz equation due to insufficient baseline data. Estimated melting temperatures for the wild type and W113F (~70 °C) were similar to the melting temperature of EC1.[22] Although we do not observe the second transition (unfolding of EC1) for E89A and W2A, as discussed below it is unlikely that these mutations affect the global stability of EC1.

In summary, both hydrophobic and ionic interactions stabilize the protein in the apo state. In the presence of calcium, the ionic interaction with E89 stabilizes the wild type and the hydrophobic interaction with W2 destabilizes the wild type. In addition, EC1 is so stable that it is not further stabilized by the binding of calcium; however, the binding of calcium manifests in a large difference in the stability of EC2.

Characterization of the Dimeric State of Protein Stocks. The dimerization state of the wild type, W2A, and E89A was assayed by native gel electrophoresis. All three proteins appeared as a single band, albeit of different sizes in

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the native gel. The wild type appeared as an extended band at a high molecular weight that is close to that of albumin. The breadth and location of the wild-type band indicate that it forms a dimer and that the monomeric and dimeric forms are in equilibrium (Figure 4A). In contrast, W2A and E89A migrated

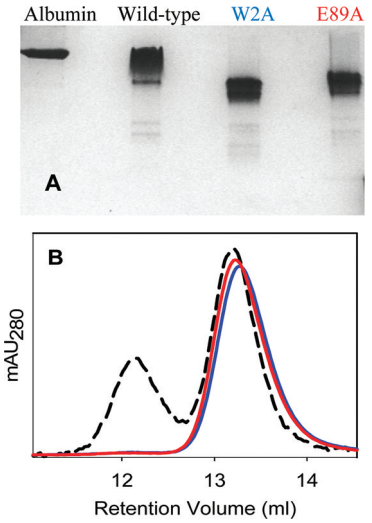

Figure 4. Assessment of the dimerization state of the wild type and mutants in the apo state. (A) Native PAGE of NCAD12 constructs. Albumin has a size and pI similar to those of the NCAD12 dimer structures. (B) Analytical SEC of the wild type (black), W2A (blue), and E89A (red) in the apo mobile phase.

further in the gel, indicating that they are smaller than the wild-type protein. These results confirm that mutations at the dimer interface weakened the dimerization ability of the protein. Similar results were observed by analytical SEC (Figure 4B). The wild type eluted as two peaks at 12.0 ± 0.1 and 13.0 ± 0.1 mL, and both W2A and E89A eluted as a single peak at 13.0 ± 0.1 mL. These elution volumes represent the dimeric (12.0 ± 0.2 mL) and monomeric (13.0 ± 0.1 mL) species.[15] These results indicate that the wild-type stock is a mixture of monomeric and dimeric species whereas W2A and E89A are monomeric.

Assembly Studies. To further characterize the effect of the mutations on dimerization, we performed analytical SEC studies. Dimerization properties were monitored at high (100–250 μM) and low (25 μM) protein concentrations in the presence of calcium (1 mM). We expected that the elution volume would decrease (more dimer) as the protein concentration was increased. This phenomenon was observed for the wild type (Figure 5), indicating that the wild-type protein forms a dimer in a protein concentration-dependent manner in the presence of calcium. In contrast, W2A eluted at the monomer elution volume, and the elution volume did not change with protein concentration (Figure 5), indicating that the W2A mutation obstructed the dimerization ability of the protein. The protein concentration-dependent change in the elution volume was moderate in E89A, indicating that the mutation decreased the dimerization affinity. In summary, the W2A mutation eliminated dimerization, and the E89A mutation decreased the dimerization affinity of the protein.

Spectral Studies. To monitor the calcium-dependent changes in the environment of W2 and W113, the intrinsic tryptophan fluorescence was monitored for the wild type, W2A, W113F, and E89A. Figure 6A compares the FL spectra for the

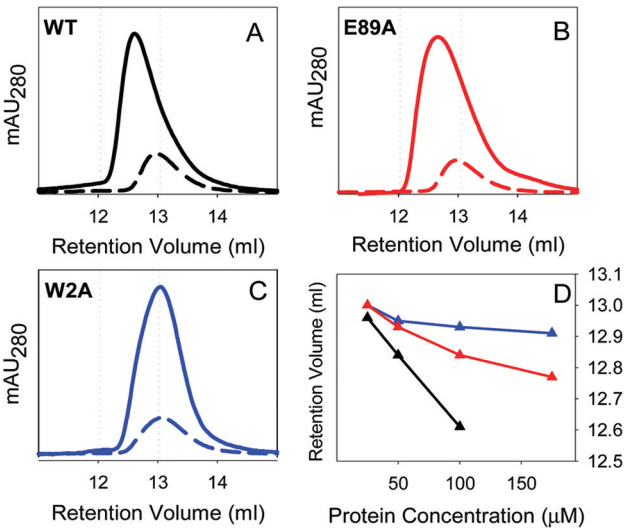

Figure 5. Analytical SEC to assess dimerization as a function of protein concentration. Chromatograms of calcium-saturated samples for each construct are shown at two concentrations. (A) Concentrated wild-type stocks (55 μM, solid line) were diluted (1:8 to 7 μM, dashed line). (B) Concentrated E89A stocks (250 μM, solid line) were diluted (1:10 to 25 μM, dashed line). (C) Concentrated W2A stocks (250 μM, solid line) were diluted (1:10 to 25 μM, dashed line). These samples were analyzed under calcium-saturated conditions (1 mM calcium). (D) Elution volume plotted as a function of protein concentration for the wild type (black), E89A (red), and W2A (blue).

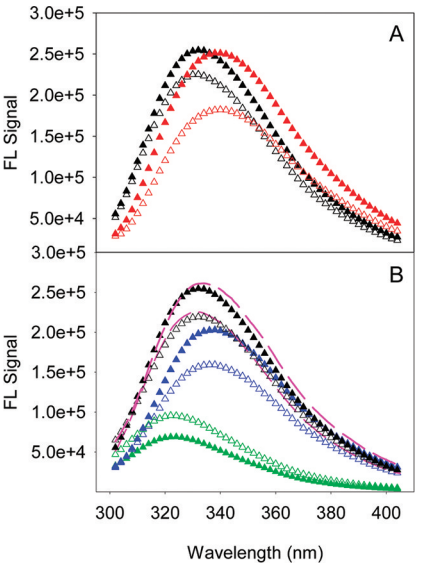

Figure 6. Fluorescence spectra as a function of calcium. Fluorescence spectra of the NCAD12 constructs are shown in the absence (empty) and presence (filled) of calcium. (A) Comparison of the emission spectra of the wild type (black) to E89A (red). (B) Comparison of the wild type (black), W2A (blue), and W113F (green). The sum of the signal from W2A and W113F is shown in the presence (pink dashed line) and absence (pink solid line) of calcium.

apo and calcium-saturated states of the wild type and E89A, the two constructs with both tryptophans. The wild type exhibited an emission maximum at 330 nm (Table 3) with greater intensity. E89A exhibited an emission maximum at 339 nm that is shifted to the red relative to that of the wild type, indicating that the loss of the ionic interaction with the N-terminus due to the mutation of E89 increased the exposure of a tryptophan

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Table 3. Summary of Fluorescence Emission Maxima in the Presence and Absence of Calcium[a]

protein	apo λ_{max}	Ca^{2+} λ_{max}
wild type	330	331
W2A	337	336
W113F	325	323
E89A	339	340

[a]The accuracy of reported emission maxima is ±1 nm. The center of mass calculations yielded different wavelengths for emission maxima, but the same spectral shifts.

residue, presumably W2. Thus, the E89A mutation opens the βA sheet and partially exposes W2. The addition of calcium does not change the emission maximum (λ_{max}) but does change the intensity of the emission, indicating that the binding of calcium changes the environment of the tryptophans, but not their exposure to solvent. These results preclude the existence of the open calcium-saturated monomer that is depicted as a possible intermediate state for the formation of the crossover dimer (Figure 1).

Next, we compared the FL spectra for the two single-tryptophan mutants, W2A and W113F, to those of the wild type. W2A exhibited an emission maximum at 336 nm (signal from W113) and W113F (signal from W2) at 325 nm (Figure 6B). These results indicate that W2 is in a less polar environment than W113 is. The emission maximum for the wild type is between those observed for W2 and W113 because the wild-type signal comprises the signal from both W2 and W113. The sum of the FL signal from W2A and W113F was approximately equal to that of the wild type, which indicates that these mutations did not significantly impact the apoprotein structure.

Studies of W113F provide information about the calcium-dependent changes in the environment of W2. The signal from W2 (W113F) was shifted to the blue by 2 nm upon addition of calcium, indicating further burial of W2 in the hydrophobic pocket (Table 3). The intensity of the W2 signal also decreased, which is the opposite direction in comparison to the three other proteins. Thus, our results provide no basis for arguing the existence of the open calcium-saturated monomer depicted in Figure 1 but rather support the opposite scenario. That is, binding of calcium to the monomer further buries W2 in the hydrophobic pocket.

To track the solvent exposure of W2 and W113, all four proteins were denatured with 8 M GHCl and the FL signal was monitored. In the native state, all four proteins had different emission maxima as discussed above. In the denatured state, all four proteins had identical emission maxima that were significantly red-shifted (355 nm, data not shown). This is an important control experiment that indicates the emission maxima for W2 and W113 do increase as they are exposed.

DISCUSSION

The adhesive dimer in cadherins is a strand-crossover structure that has critical hydrophobic and ionic interactions that have been proposed to be essential for its formation. An interesting aspect of this system is that these noncovalent interactions are the same in the closed monomer and in the closed strand-crossover dimer. Thus, if there is no net change in the noncovalent interactions, what drives the formation of dimer and why is dimer formation dependent upon the binding of calcium? We find that while the ionic interaction involving E89 is important for the general stability of the adhesive dimer, the hydrophobic interaction involving W2 is the source of the calcium requirement for adhesive dimer formation. We present data here that provide a thermodynamic rather than structural argument for the calcium dependence of dimer formation.

Energetic Evidence of the Calcium-Induced Strain Model. A model that summarizes the thermodynamic evidence of calcium dependency in strand-crossover dimer formation is shown in Figure 7. According to this model, in the closed apo monomer, the βA sheet is associated with EC1 such that W2 is docked in its own hydrophobic pocket and E89 is interacting with the N-terminus. The binding of calcium at the EC1−EC2 interface creates strain in the closed monomer, specifically in the interaction between the hydrophobic pocket and W2. The calcium-dependent strain originates from the only direct connection between the βA sheet and calcium-binding E11. This strain is relieved upon formation of the strand-crossover dimer.

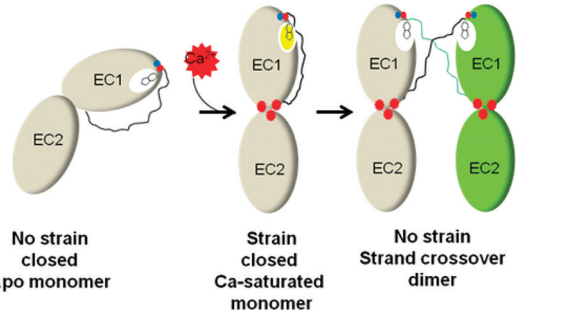

Figure 7. Model for calcium-induced dimerization. Binding of calcium creates strain in the hydrophobic pocket (yellow circle) of the closed monomer, which leads to formation of the dimer in N-cadherin. The noncovalent interactions in the closed structure are illustrated: the hydrophobic interaction between the docked W2 and the hydrophobic pocket in EC1 and the ionic interaction between the N-terminus (blue circle) and the side chain of E89 (red circle).

The relative disposition of the modular domains is sensitive to the level of calcium. Pokutta et al.[24] showed more than 15 years ago that in the apo state the modular domains are collapsed, forming a somewhat condensed structure in which the interactions between the modular domains are nonspecific and flexible. Thus, in the absence of calcium, the wild type is flexible and there is little strain at the dimer interface. Upon addition of calcium, the modular domains extend to form a rigid curved structure.[1,3,24] Conformational rigidity and the change in disposition between the modular domains induced by the binding of calcium create strain in the hydrophobic interaction in the wild type, leading to the ~2 kcal/mol free energy differences between the wild type and W2A. Recent molecular dynamics simulations of the closed calcium-saturated structure of epithelial cadherin led to similar conclusions.[25] The calcium-induced strain model is supported by the following thermodynamic and solution assembly evidence.

Hydrophobic Interactions between W2 and the Hydrophobic Pocket. Tryptophans have a distinctive fluorescence emission that is relatively strong and very sensitive to the environment of the indole ring providing us with a convenient spectroscopic probe of the docked and exposed positions of W2. First, we observed that the FL signal intensity changed upon addition of calcium, indicating that the binding of calcium changed the environment of W2. This observation

suggests that upon addition of calcium the βA sheet reorganizes, which manifests as a change in the intensity of the FL signal. Second, the FL signal from W113F (signal from W2) is slightly shifted to the blue upon binding of calcium, indicating further insertion of W2 into the hydrophobic pocket. Perhaps this is spectroscopic evidence of the origin of the strain in the hydrophobic pocket.

The strongest evidence of calcium-induced strain in the monomer is from the stability studies of the W2A mutant. These studies imply that W2 in the wild type stabilizes the protein in the apo state by ~0.5 kcal/mol and destabilizes the wild type in the calcium-saturated state by ~2 kcal/mol. The binding of calcium causes conformational strain in the interactions between W2 and the hydrophobic pocket in the wild type, which manifests as a decrease in stability. Thus, the W2−hydrophobic pocket interaction confers calcium sensitivity to the formation of the strand-crossover dimer. This is the first spectral and thermodynamic evidence of the role of W2 in the calcium dependency of dimer formation.

Our thermodynamic data for calcium-dependent conformational strain in the monomer are supported by recent molecular dynamics (MD) simulations by Vendome et al.[25] They created a model of the closed monomer of epithelial cadherin from PDB entry 1ff5[8] and measured the length of the βA sheet in the minimized structure in the presence and absence of calcium. They predict that the length of the βA sheet increases by 2.5 Å upon binding of calcium. This increase in the length of the βA sheet and our spectroscopic evidence of the further burial of W2 are structural origins for the 2 kcal/mol penalty upon binding of calcium to the wild type.

It is interesting to note that the W2A mutation in EC1 has an impact on the stability of EC2, indicating that there is active communication between domains. This was not unexpected on the basis of our studies of the isolated domains. The sensitivity of the stability of EC2 to its context was first demonstrated in studies of the effect of the adjacent seven-residue linker segments on the isolated EC2 domain of epithelial cadherin.[26] We found that the acidic linker segments that connect EC2 to EC1 and EC2 to EC3 destabilized the core EC2 domain because of electrostatic repulsion. Further destabilization of EC2 by EC1 was demonstrated in studies of the two-domain construct of epithelial cadherin (ECAD12), which also showed a clear biphasic unfolding profile with an ~20 °C decrease in the transition temperature for EC2 in the two-domain construct compared to that of isolated EC2.[27] These studies clearly demonstrate how interdomain interactions in the apo state impact the stability of EC2. Interestingly, unlike EC2, EC1 is remarkably insensitive to calcium binding (Figure 3). The similarity of the second transition to melting profiles of isolated EC1 domain indicates that the stability of EC1 is not influenced by EC2, or by the linker segments. The stability of EC1 constructs was not influenced by the addition of salt, indicating that in spite of the acidic pI of EC1, electrostatic repulsion is not a major factor in its stability (data not shown). In summary, the effects of the mutations in EC1 are not manifesting in EC1 but rather are reflected in EC2 likely through changes in electrostatic interactions at the EC1−EC2 interface involving E11.

Assembly studies showed that the W2A mutation obstructed the dimerization ability of the protein. Our chromatographic studies are in agreement with studies by a number of other groups that have shown W2 is required for dimerization.[4,7,11,28]

Ionic Interactions between E89 and the N-Terminus. The carboxylic acid group on the side chain of E89 participates in an ionic interaction with the amine at the N-terminus of the protein. Antibody studies by Harrison et al.[11] illustrated that the adhesive dimer requires interactions with E89 and a properly processed N-terminus. These observations were later supported by structural studies.[2,3] Thermal unfolding studies with E89A showed that the E89−N-terminus interaction stabilizes both the closed apo and calcium-saturated monomer by ~0.5 kcal/mol. Because the stabilizing effect of this ionic interaction is the same in both the apo and calcium-saturated states, this interaction is not a contributor to the requirement of calcium for adhesive dimer formation. It was also interesting that we had clear evidence of dimer formation by E89A. E89A forms a dimer, but it is less stable than the wild-type dimer. Thus, the E89−N-terminus interaction has an overall stabilizing effect in the closed structures, monomer or dimer, but is not strictly required for dimer formation.

Intermediate State in the Formation of the Strand-Crossover Dimer. Several crystallographic studies have shown the structure of the strand-crossover dimer in which W2 is docked in the hydrophobic pocket of the partner protomer.[1,2] To form the strand-crossover dimer, each protomer must undock tryptophan from its own hydrophobic pocket and dock it into its neighbor's. If the open structure is formed, it manifests as a shift in the FL emission maximum. Surface accessibility studies suggest that W2 is completely buried in the hydrophobic pocket. FL spectral studies showed the FL signal intensity changed as a function of calcium but there was no red shift in emission maximum in the four proteins. This suggests that calcium binding is creating conformational change in the protein but is not opening the βA strand. Calcium-dependent conformational changes have been noted in a number of studies.[24,29] Although our analytical SEC studies unambiguously demonstrated the formation of the dimer, there was no spectroscopic evidence of an open intermediate structure. One possible explanation is that the strand-crossover dimer forms via the "initial encounter complex",[12] and that this complex involves the strand−dimer interface such that the open structure is never exposed to solvent. In summary, our studies showed calcium-dependent conformational change and formation of dimer but not the open intermediate structure. The experimental evidence of the intermediate state in the formation of the strand-crossover dimer for the wild type in solution is still to be discovered.

Taken together, for the first time, our data provide a thermodynamic argument for the calcium dependency for strand-crossover dimer formation in N-cadherin. Studies presented here show that calcium binding is energetically unfavorable for the monomeric conformation because of strain in the W2−hydrophobic pocket interaction. The direct link between calcium binding to Ell and the βA sheet creates this strain. To overcome this unfavorable condition, N-cadherin forms a dimer.

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■ ABBREVIATIONS

apo, calcium-depleted; CD, circular dichroism; EC, extracellular domain; EC1, extracellular domain 1 of NCAD12; EC2, extracellular domain 2 of NCAD12; EGTA, ethylene glycol tetraacetic acid; E89A, mutation of glutamate 89 to alanine in NCAD12; GHCl, guanidine hydrochloride; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NCAD12, neural cadherin domains 1 and 2 (residues 1−221); PAGE, polyacrylamide gel electrophoresis; PDB, Protein Data Bank; SDS, sodium dodecyl sulfate; SEC, size exclusion chromatography; T_m, melting temperature; W2A, mutation of tryptophan 2 to alanine in NCAD12; wild type, wild-type NCAD12.

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