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Stability studies of extracellular domain two of neural-cadherin

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ABSTRACT

Neural- (NCAD) and epithelial- (ECAD) cadherin are calcium-dependent cell-adhesive molecules, and are localized at excitatory and inhibitory synapses respectively. They play an important role in synaptogenesis, synapse maintenance and plasticity. The extracellular region plays a critical role in cadherin-mediated cell adhesion, and has five tandemly repeated ectodomains (EC1-EC5). Calcium binding is required for dimer formation between first two N-terminal domains (EC1-EC2). Despite similarity in the primary structure, the extracellular domains of NCAD and ECAD have different intrinsic stability, dimerization affinity and kinetics of disassembly. To investigate the origin of these differences, we are characterizing the modular domains individually. Here, we report studies of NCAD2, EC2 of NCAD. This domain is important for calcium binding and is the physical linkage between the dimerization interface in EC1 and the membrane proximal modular domains. Thermal-denaturation studies show that NCAD2 is less stable than ECAD2 and less influenced by the adjoining 7-residue, N- and C-terminal linker segments. In addition the NCAD2 constructs are less influenced by added salt. This difference is likely due to variation in the overall number and distribution of charges on these anionic proteins. Our studies indicate that despite their sequence similarity and apparently passive role in adhesive dimer formation, EC2 of E- and N-cadherins are distinctly different and may contribute to the differences in energetics and kinetics of dimerization.

1. Introduction

Classical cadherins are calcium-dependent cell adhesion proteins that are critical for solid tissue formation. The N-terminal extracellular region of cadherins comprises 5 modular domains and is the site for the adhesive interaction between identical cadherins on adjacent cells. Members of the classical cadherin family are named according to the tissue in which they predominate, but can occur in other tissues. For example, neural-cadherin (NCAD) is found at excitatory synapses where calcium levels fluctuate, whereas epithelial-cadherin (ECAD) is found at inhibitory synapses where the extracellular calcium concentration is uniformly high [1–4]. This distinct localization implies that their properties are appropriate for the needs of that specific cell–cell junction. The first two modular domains of neural- and epithelial cadherins, NCAD12 and ECAD12, are considered minimal functional adhesive units containing both the adhesive dimer interface and an intact calcium binding pocket. Although their calcium binding properties are identical [5], a number of other properties of these constructs are different. NCAD12 and ECAD12 have different stabilities [6], dimerization affinity [7] and kinetics of dimer dissociation [6]. In order to understand the origin of energetic and kinetic differences between NCAD and ECAD, we have undertaken studies of their modular extracellular domains.

This report characterizes NCAD2, the second EC-domain of NCAD. We have been particularly interested in EC2 as a contributor to the energetic and kinetic differences between the two-domain constructs ECAD12 and NCAD12. EC2 is essential for adhesive dimer formation by playing a critical role in forming the calcium binding pocket at the EC1- EC2 interface (c.f. [8]). Dimer formation is sensitive not only to mutations in the 3 calcium binding sites that are at the EC1–EC2 interface [5,9–12], but also to those at the EC2–EC3 interface [13] indicating that the signal of calcium ligation is conveyed through EC2 to the dimer interface in EC1 and this communication is essential for adhesion. In addition, EC2 has been proposed to be the site of the *lateral* interaction in which EC1 from one protomer interacts with EC2 from a partner protomer. This lateral interaction was shown to be important for the integrity of adherens junctions [14]. Foremost, recent reports have documented a dimeric structure, the X-dimer that enhances the disassembly of the strand-crossover dimer [15,16]. There are three critical noncovalent interactions between protomers at the X-dimer interface, all of which involve residues in EC2, including N143, D138 and E199 of ECAD2. Interestingly, none of the X-dimer interactions in which these residues participate in ECAD2 are conserved in NCAD2 and therefore may contribute to the kinetic differences noted for the strand-crossover dimers of ECAD and NCAD. Since Adherens Junctions must assemble and disassemble to function properly, these fundamental differences in the X-dimer interface in

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EC2 may impact the specific localization of these proteins. Thus, because of its importance as a physical and energetic link between calcium binding and dynamics of dimerization, we report here studies of the calcium-dependent stability of EC2 of NCAD.

Comprehensive studies of EC2 of ECAD have demonstrated several important attributes. ECAD2 is only 106 amino acids in size with no internal disulfide linkages, yet is remarkably stable with a free energy of unfolding at 25 °C of 6 kcal/mol ($T_m = 54$ °C) [17]. Secondly, the stability of the core domain is altered by addition of the linker segments, short 7-residue acidic peptides that link EC2 to EC1 (linker 1) and link EC2 to EC3 (linker 2) [18] (Fig. 1A). The melting temperature decreased by 9 °C ($\Delta\Delta G = -2.0$ kcal/mol) with addition of both linker segments. Further, salt-dependent stability studies showed that the origin of the destabilization by the acidic linker segments was due to unfavorable electrostatic interactions with the acidic residues in the loops that connect the β-sheets in the EC-modules.

The studies presented here are part of an ongoing project to characterize the modular extracellular domains of cadherins to investigate how their properties are tailored to contribute to calcium-dependent dimerization. Here, we present salt- and calcium-dependent stability studies of NCAD2. We find that NCAD2 is less stable than ECAD2, but is also less stabilized by addition of salt. Review of structural data on these domains show that there is a profound difference in the distribution of acidic residues on the surface of these EC2 constructs, which may contribute to the differences in stability and the influence of salt upon their properties. Ultimately, these fundamental differences may play a role in the energetics and dynamics of dimerization.

2. Methods and materials

2.1. Cloning, expression and purification

NCAD2 (residues E107 to T214) and L1-NCAD2-L2 (residues D100 to P221) were subcloned from a cDNA mouse NCAD clone kindly provided by Prof. L. Shapriro (Columbia University) using a Xa/LIC cloning kit (Novagen) into a pET31b vector. Proteins were expressed in BL21(DE3) bacterial cells. Both EC2 constructs were soluble and were purified from the supernatant of the lysed whole cells. Details of the cloning, expression and purification procedure are described elsewhere [6].

2.2. Characterization of purified proteins

Protein purity was assessed with SDS-PAGE and molecular mass was determined by mass spectrometry at the Molecular Analysis Facility at Stanford University. The extinction coefficient was determined experimentally [19] and found to be $10800 \pm 900\ M^{-1}\ cm^{-1}$ for NCAD2 (one tryptophan and four tyrosines).

Fig. 1. Comparison of EC2 of ECAD and NCAD. (A) The sequences of L1-NCAD2-L2 (bottom: red) and L1-ECAD2-L2 (top: black) of mouse cadherin were compared using LALIGN (identical (:), similar (.) [20]). Linker segments are underlined. At pH 7.0, L1-NCAD2-L2: pI 4.35, charge −8.8; NCAD2: pI 4.48, charge −5.8; L1-ECAD2-L2: pI 4.27, charge −11.8; ECAD2: pI 4.35, charge −8.8. Yellow residues chelate calcium. Blue are in the X-dimer interface [15], and green are in the lateral-dimer interface [14].

2.3. Temperature-induced denaturation as a function of calcium and salt

Estimates of stability were determined by thermal denaturation of native proteins monitored with an AVIV 202SF Circular Dichroism (CD) Spectrometer. CD signal was measured for solutions with 5 μM protein at 225 nm through a temperature range of 15–85 °C ramped at 1 °C/min (30 s equilibration, 5 s acquisition time). Apo-buffer conditions were 2 mM HEPES and 140 mM NaCl at pH 7.4. Calcium-saturated conditions had 5 mM calcium added. To observe the reversibility of the unfolding transitions, the CD signal was monitored as the denatured samples were cooled back to 15 °C. To observe the effect of NaCl concentration, proteins were in 10 mM sodium phosphate, and varying added salt concentrations from 0, 10 mM, 50 mM, 140 mM, 500 mM, and 1 M added NaCl at pH 7.4. Data were fit to the Gibbs-Helmholtz equation (Eq. (1)) with linear folded and unfolded baselines using Igor Pro as described elsewhere [17] to provide estimates of the denaturation temperature (T_m) and enthalpy (ΔH_m).

$$\Delta G^{o'} = \Delta H_m\left(1-\frac{T}{T_m}\right) - \Delta C_p\left[T - T_m - ln\left(\frac{T}{T_m}\right)\right] \quad (1)$$

The value for ΔC_p was fixed to the value of determined for ECAD2 (1 kcal/(Kmol)) [17]. The Gibbs free energy change ($\Delta G°'$) at 25 °C was calculated from fitted values of T_m and ΔH_m.

3. Results

Here, we present a systematic study of the effect of the adjoining linker segments, calcium binding and salt concentration on the stability of isolated EC2 of NCAD that compare directly to identical studies of ECAD2 constructs. These are part of a larger effort to determine the fundamental properties of EC2 in the calcium dependence of the dynamics of lateral and adhesive dimer formation and disassembly. Even though EC2 is not the site of the strand-crossover dimer interface, EC2 is of interest because of its role in communicating adhesion and calcium binding between the membrane distal and proximal regions of the protein, and as the site of both lateral and X-dimer interactions. Thus, properties of EC2 have mechanistic implications in the function of cadherins, in general, and dynamics of adherens junctions, in particular.

3.1. Sequence comparison

As a means to illustrate the similarities and differences between NCAD2 and ECAD2, the sequences of L1-ECAD2-L2 (top) and L1-NCAD2-L2 (bottom) were aligned (Fig. 1). According to LALIGN [20], there is 73% identity and similarity between NCAD2 and ECAD2, with the difference in the charged residues as the most notable difference. NCAD2 has lower net charge than ECAD2, with a total charge at pH 7 of −5.8 (11 acidic and 6 basic residues) for NCAD2 as compared to −8.8 for ECAD2 (15 acidic and 7 basic residues). Second, although NCAD2 has more hydrophobic residues (34%) than ECAD2 (31%), the number of buried hydrophobic residues (<5% exposed surface area; GetArea [21]) in the two constructs is equivalent.

Linkers are short 7 residue segments that connect each of the 5 modular domains to the next. The consensus linker sequence in cadherins is DXNDNXP [22]. Linker 1 and linker 2 together add one basic and 4 acidic residues to EC2. The linker segments contain residues that are essential for binding of calcium, but the linkers alone do not create intact calcium binding pockets at the termini of the domain. The linker 1- EC2 interface has only the residues that

comprise site3 in the calcium binding pocket at the EC1- EC2 interface. The EC2- linker 2 interface has only the residues that comprise site1 for the EC2- EC3 interface. Thus, the construct with linkers, L1-NCAD2-L2 would be expected to bind calcium.

A similar alignment of EC1 modules of mouse ECAD and NCAD yields 84% sequence identity or similarity leading to an overall 81% identity and similarity for the two-domain constructs NCAD12 and ECAD12. Thus, even though the strand-crossover dimer interface is in EC1, the EC2 domains show the greatest heterogeneity.

3.2. Structural comparison and difference in ionic character

As a further comparison of ECAD2 and NCAD2, we present a surface map of charged residues on the proteins. NCAD2 and ECAD2 structures were minimized and then aligned (Fig. 2). The 3-D structures of the two constructs are remarkably similar with only 0.72 Å r.m.s.d. difference in the α-Carbon positions. The greatest structural difference in the two structures is in the loop regions at the EC1-EC2 and EC2-EC3 interfaces.

Opposing faces of the EC2 structures are presented and arbitrarily called "front" and "back". Notice that the front view of both EC2 domains displays a cluster of charged residues at the far right and left ends. These residues are important for chelating the calcium that binds at the EC1–EC2 interface (right) and the EC2–EC3 interface (left). The adjoining linker segments, each containing 2 acidic amino acids, would be directly adjacent to these acidic regions in the L1-EC2-L2 constructs. Also, notice that there is a similar frequency of charged amino acids on the front side for NCAD2 and ECAD2, even though the distribution differs. On the back of the domains, there is a significantly less charge on NCAD2 and more on ECAD2.

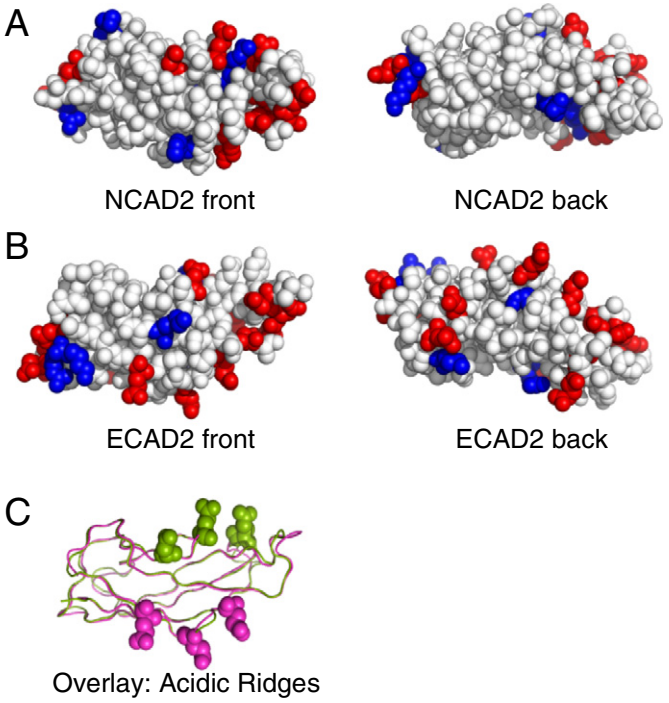

Fig. 2. Comparison of charge distribution on the surface of NCAD2 and ECAD2. The portion of the structure corresponding to ECAD2 and NCAD2 were excised from the crystal structure (PDB ID: ncj1; [30] and PDB ID: 1edh; [8], respectively) and minimized using AMBER [31] to relieve the effect of the linker segments and EC1. These minimized structures were then aligned using the superimpose utility in PyMOL [32]. (A) NCAD2 and (B) ECAD2 are shown with acidic (red) and basic (blue) residues highlighted. Figures on right are rotated 180° vertically (horizontal axis of rotation). (C) The aligned backbone structure is traced with a ribbon. The acidic amino acids in the "acidic ridge" are shown for NCAD2 (green: from left to right E170, D167 and E196) and ECAD2 (pink: from E114, E111 and E107).

Of interest is a longitudinal ridge of acidic residues that is apparent on both constructs and is highlighted in Fig. 2C. The residues that are involved differ between the ECAD2 and NCAD2, and are on opposite faces of the domain. The acidic ridge of ECAD2 involves residues E115, E111, E107, and that for NCAD2 involves D170, E167 and E196. The side chains of the "acidic ridge" residues on ECAD are 84% or more accessible to solvent [21]. This is in contrast to those of NCAD where the side chains of D170, E167 and E196 are exposed 23%, 91% and 60%, respectively. In addition, there is an arginine (R146) that has an electrostatic interaction with E196. There are two major observations that can be made about this difference. First, the acidic ridge residues are all on the βA-sheet in ECAD2, leading to a relatively small impact of the electrostatic repulsion in the ridge on the tertiary structure of the construct. This is in contrast to the acidic ridge residues in NCAD2 which are on two different sections of the protein. D170 and E167 are in the loop connecting the βB- and βC-sheets, and E196 is in the loop connecting the βF- and βG-sheets. Thus, while electrostatic repulsion between the acidic ridge residues may destabilize the protein, the ionic bond between R140 and E196 could serve to reduce this effect. These differences in charge distribution between NCAD2 and ECAD2 may have broad ramifications in intermolecular interactions, but is beyond the scope of the studies reported here.

3.3. Calcium-dependent thermal-stability studies

Thermal denaturation of NCAD2 and L1-NCAD2-L2 was performed to assess the global stability of EC2 and how it is affected by adjoining segments and calcium. The normalized CD signals obtained from the NCAD2 and L1-NCAD2-L2 temperature denaturation studies are compared in Fig. 3 for the apo buffer condition. The T_m resolved from the fits to a 2-state model and calculated values for $\Delta G^{\circ\prime}$ at 25 °C are reported in Table 1 (140 mM NaCl). The resolved value for enthalpy change for all apo-NCAD2 data was 67 ± 3 kcal/mol and for L1-NCAD2-L2 data was 61 ± 3 kcal/mol. The solid lines passing through the data were simulated based on resolved parameters. NCAD2 is more stable in apo conditions compared to L1-NCAD2-L2; the addition of the adjacent linkers segments to NCAD2 destabilized the domain by 4.4 °C ($\Delta\Delta G^{\circ\prime} = -1.0$ kcal/mol).

The addition of 5 mM calcium stabilized NCAD2 by less than 1 °C (no change in $\Delta G^{\circ\prime}$) but had a significant effect on L1-NCAD2-L2 (6.5 °C increase in T_m, $\Delta\Delta G^{\circ\prime} = 1.1$ kcal/mol). We expected this difference since the L1-NCAD2-L2 construct contains the linker segments thereby completing the site1 at the EC2- linker 2 interface and site3 at the linker 1- EC2 interface [8,22,23]. Thus, calcium should stabilize L1-NCAD2-L2 if calcium will bind to the intact site in the absence of the binding to the other two incomplete sites. It is also

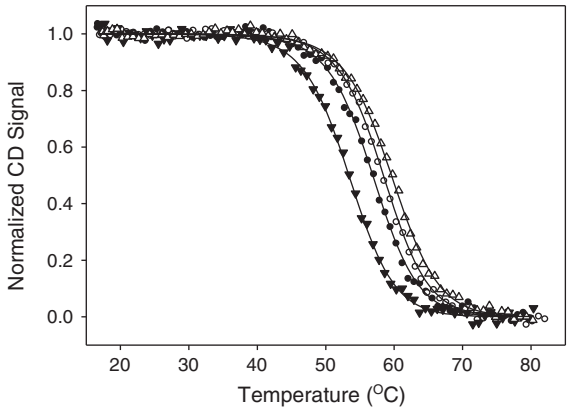

Fig. 3. Calcium-dependence of thermal denaturation of NCAD2 constructs. CD signal at 225 nm was normalized for native and denatured baselines and plotted against temperature for L1-NCAD2-L2 (Δ) and NCAD2 (○) in the apo (filled) and 5 mM calcium-added (open) states.

Table 1
Thermodynamic parameters resolved from fits to data from temperature denaturation of NCAD2 constructs in different calcium concentrations[a].

Conditions	NCAD2		L1-NCAD2-L2	
	T_m	$\Delta G°'$	T_m	$\Delta G°'$
Apo	58 ± 1	5.0 ± 0.5	53.7 ± 0.7	4.0 ± 0.4
5 mM Ca^{2+}	58.7 ± 0.4	4.8 ± 0.3	60.2 ± 0.8	4.9 ± 0.4

[a] T_m in °C and $\Delta G°'$ in kcal/mol. Experiments performed in triplicate. ΔC_p was fixed to 1 kcal/(Kmol). Reported error in T_m is from simultaneous analysis of triplicate data sets. $\Delta G°'$ was calculated from Eq. (1) based on fitted parameters.

interesting to note that the addition of calcium counteracts the destabilizing effect of the linkers on apo NCAD2 and restores the construct with linkers to the stability of the core domain.

3.4. Salt-dependent studies

Since NCAD2 and L1-NCAD2-L2 are acidic and highly charged proteins, we suspected that electrostatic repulsion may play a role in their overall stability. To study the role of electrostatics in the stability of NCAD2 constructs, we performed thermal-denaturation experiments as a function of added NaCl. The normalized CD signals are plotted in Fig. 4A and B. The values for $\Delta G°'$ at 25 °C were calculated from resolved values for T_m and ΔH_m and plotted against the concentration of NaCl in Fig. 4C. The T_m of both NCAD2 constructs increased as the salt concentration increased from 0 mM to 1 M added salt (ΔT_m is 8 °C for NCAD2 and 12 °C for L1-NCAD2-L2). In spite of the modest increase in T_m for NCAD2, upon consideration of ΔH_m there was no obvious salt-dependent increase in stability indicating that the core EC2 structure is not significantly destabilized by electrostatic repulsion. In contrast, L1-NCAD2-L2 showed an increasing stability with added salt up to 500 mM NaCl, indicating that the acidic linker segments destabilize the core domain. Data from similar studies on ECAD2 and L1-ECAD2-L2 are shown for the purpose of comparison [17,18]. Note the following points. First, the core domain ECAD2 is more stable at all salt concentrations than ECAD2 construct with linkers, and more stable than either of the two NCAD2 constructs. Second, the addition of linkers has a greater destabilizing effect on ECAD2 than linkers have on NCAD2. Third, it is clear that the salt dependent increase in stability for ECAD2 constructs is significantly greater than seen for the NCAD2 constructs.

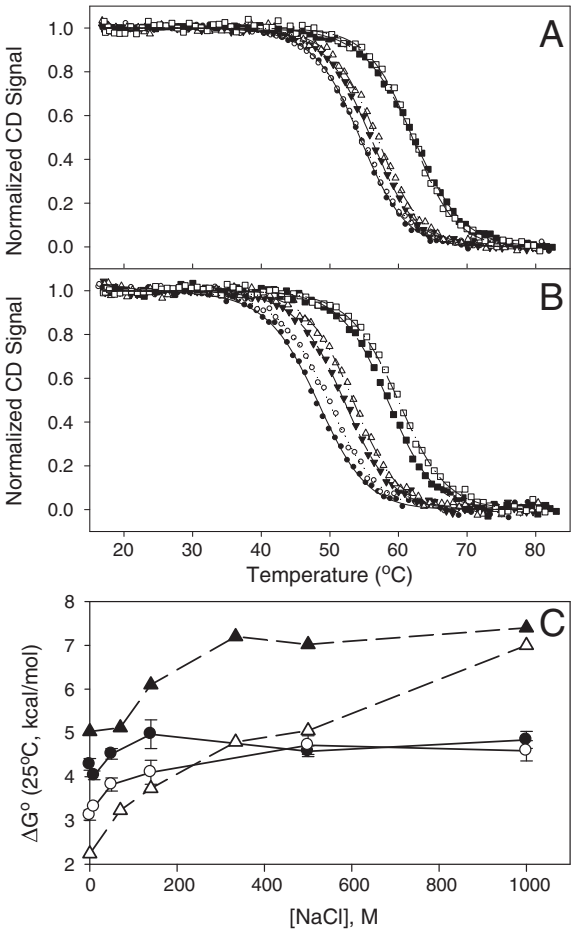

Fig. 4. Salt dependence of thermal denaturation of NCAD2 constructs. (A) NCAD2, (B) L1-NCAD2-L2 at 10 (●), 50 (○), 100 (▼), 150 (Δ), 500 (■) and 1000 (□) mM added NaCl. (C) Values for $\Delta G°'$ calculated from resolved values for T_m and ΔH_m from thermal-denaturation of NCAD2 (●) and L1-NCAD2-L2 (○) plotted versus the concentration of NaCl added to the buffer. The data for ECAD2 (▲) and L1-ECAD2-L2 (Δ) are for comparison purposes [18].

4. Discussion

Cadherins participate in adhesions that indirectly link the actin cytoskeletons of neighboring cells. Dimer formation between EC1 domains is communicated through the 5 extracellular modules to the cytoplasmic domain where interactions with catenins and other cytoplasmic factors regulate cell physiology. A long term interest of our laboratory has been how the differences in affinity and kinetics of adhesive dimer disassembly between NCAD and ECAD correlate with their physiological localization. Since these biophysical properties pertain to the EC-domain and its component modules, we are interested in fundamental properties of the EC-modules that impact function. Since EC2 is an intermediary module that links the adhesive interface in EC1 to the membrane proximal domains, calcium- and salt-dependent stability studies of EC2 provide insight into the noncovalent forces that are relevant to its role in the linkage between calcium binding and the dynamics of dimerization. Recent literature highlights the essential role of EC2 as a component of the lateral dimer [14], and as a critical player in the dynamics of dimer disassembly as the site of the X-dimer interface [15,16]. Here, we report that ionic strength-dependent differences between ECAD2 and NCAD2 correlate with the difference in their ionic character.

The findings in this paper are summarized as follows. The construct with linkers, L1-NCAD2-L2, is less stable than NCAD2 and more stabilized by the addition of salt. Each linker segment contains two aspartate residues (Fig. 1). Thus, one would expect increased electrostatic repulsion for the construct with the linkers, L1-NCAD2-L2, due to repulsive forces between the anionic linker regions and the clusters of acidic amino acids in the loop regions at the interdomain interfaces. Increased salt concentration effectively adds Na^+ ions that screen negative charges in the protein, decreasing electrostatic repulsion and increasing the stability of the protein. Calcium stabilizes L1-NCAD2-L2 since the linkers provide the residues for one of the 3 calcium-binding sites at each interface (site3 at the Linker 1- EC2 interface and site1 at the EC2- linker 2 interface).

Results from these studies on NCAD2 constructs can be compared directly to an identical set of studies on ECAD2 constructs where the general trends are similar, but the details differ significantly. ECAD2 had lower resolved values for T_m and greater values for ΔH_m than the NCAD2 constructs leading to significantly higher calculated values for $\Delta G°'$ at 25 °C (140 mM NaCl: $\Delta G°'$ (apo-NCAD2) = 5.0 kcal/mol, $\Delta G°'$ (apo-ECAD2) = 6.3 kcal/mol [17]; Fig. 3C). Further, the linker segments have a larger destabilizing effect on ECAD2 than seen for NCAD2 [18]. This is likely due to the fact that NCAD2 carries less negative charge overall than does ECAD2 (Fig. 1), and therefore there is less electrostatic repulsion by the linkers at pH 7. Thus, due to increased electrostatic repulsion in ECAD2 constructs, one would

expect to see a larger linker effect and increased sensitivity to salt than seen in NCAD2, which is what was observed.

It is clear that if the addition of salt stabilizes the protein that it must be screening repulsive ionic forces rather than attractive forces. This general phenomenon has been documented for anionic proteins in the literature. Maldonado et al. studied this phenomenon in detail for apoflavodoxin [24]. They note that in apoflavodoxin, longer range repulsive interactions are offset by shorter range attractive interactions. Studies of mutants of a Fibronectin Type III domain showed that loss of charge increased the stability of the protein as did the screening effects of increased salt concentration [25]. A similar phenomenon was noted in a cationic protein, Histone 2A-2B where deletion of the basic N-terminal tail significantly increased the stability of the dimer [26], and added salts decreased electrostatic repulsion [27].

Inspection of the distribution of charged residues of ECAD2 and NCAD2 reveals several interesting trends. First, there are clusters of negatively charged amino acids at the interdomain- calcium binding interfaces. This is expected since the groups that chelate calcium ions in the interfacial region are acidic or carbonyl groups. Second, a ridge of acidic amino acids runs longitudinally from the midpoint toward the EC2- linker 1 interface. Although both NCAD2 and ECAD2 have this acidic ridge, they are asymmetrically distributed on the surface such that they are on opposite faces of the domain (Fig. 4). Third, there are significantly more charged residues on the "back" of ECAD2 than there are on NCAD2. These differences in the number and distribution of charges may play a role in cooperativity between domains in a single molecule and between molecules in an adhesive dimer. This observation offers a possible explanation for the difference in the energetic and kinetic differences in the NCAD and ECAD.

Recent studies in our laboratory have shown that the EC1 module for NCAD is remarkably stable with a melting temperature of ~70 °C. This domain is not stabilized by calcium either in the isolated NCAD1-L1 construct, or in the two-domain construct (NCAD12) indicating that calcium dissociates before the domain unfolds [28]. NCAD2, however, is impacted by the presence of the linker segments (Fig. 3), and further impacted by EC1 in the NCAD12 construct in the apo state ($\Delta T_m = -13$ °C; $\Delta\Delta G^{\circ\prime} = -1.4$ kcal/mol [28]) indicating that EC2 is very sensitive to its context. In the presence of calcium, contrary to EC1, the stability of EC2 is profoundly affected by calcium binding ($\Delta T_m = 2$ °C; $\Delta\Delta G^{\circ\prime} = 1.5$ kcal/mol [6]). Similar behavior was observed for the EC2 domain of ECAD; ECAD2 is destabilized by the linker segments [18] and further destabilized in ECAD12 [29]. Thus, ample evidence exists that EC2 is more responsive to its environment and interdomain interactions than is EC1.

Even though it is not the site of the strand-crossover dimer interface, interest in the fundamental characteristics of EC2 is because of its important role in communicating adhesion and calcium binding between the C-terminal proximal regions to the distal dimer interface, and as the site of both lateral and X-dimer interactions. Thus, properties of EC2 have mechanistic implications in the function of cadherins in general and dynamics of adherens junctions in particular. These studies point to the complexity of the modular system in creating a cooperative whole that confers equilibrium and kinetic properties on cadherins that tailor them to their specific physiological niche.

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