Calpain Activation by Cooperative Ca\textsuperscript{2+} Binding at Two Non-EF-hand Sites*

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The active site residues in calpain are mis-aligned in the apo, Ca\textsuperscript{2+}-free form. Alignment for catalysis requires binding of Ca\textsuperscript{2+} to two non-EF-hand sites, one in each of the core domains I and II. Using domain swap constructs between the protease cores of the \( \mu \) and \( m \) isoforms (which have different Ca\textsuperscript{2+} requirements) and structural and biochemical characterization of site-directed mutants, we have deduced the order of Ca\textsuperscript{2+} binding and the basis of the cooperativity between the two sites. Ca\textsuperscript{2+} binds first to the partially preformed site in domain I. Knockout of this site through D106A substitution eliminates binding to this domain as shown by the crystal structure of D106A \( \mu \)–I–II. However, at elevated Ca\textsuperscript{2+} concentrations this mutant still forms the double salt bridge that links the two Ca\textsuperscript{2+} sites and becomes nearly as active as \( \mu \)–I–II. Elimination of the bridge in E333A \( \mu \)–II has a more drastic effect on enzyme activity, especially at low Ca\textsuperscript{2+} concentrations. Domain II Ca\textsuperscript{2+} binding appears essential, because Ca\textsuperscript{2+}-coordinating side-chain mutants E392R and D333A have severely impaired \( \mu \)–II activation and activity. The introduction of mutations into the whole heterodimeric enzyme that eliminate the salt bridge or Ca\textsuperscript{2+} binding to domain II produce similar phenotypes, suggesting that the protease core Ca\textsuperscript{2+} switch is crucial and cannot be overridden by Ca\textsuperscript{2+} binding to other domains.

Calpains are cystolic cysteine proteases that show a strict Ca\textsuperscript{2+} requirement for activity (1–2). They have been implicated in many Ca\textsuperscript{2+} signaling processes, including the cell cycle, cell death, cell motility, and associated cytoskeletal remodeling, as well as yet to be defined critical steps of embryonic development (3–8). In addition, they help regulate more specialized functions such as the basis of the cooperativity between the two sites. Ca\textsuperscript{2+} binds first to the partially preformed site in domain I. Knockout of this site through D106A substitution eliminates binding to this domain as shown by the crystal structure of D106A \( \mu \)–I–II. However, at elevated Ca\textsuperscript{2+} concentrations this mutant still forms the double salt bridge that links the two Ca\textsuperscript{2+} sites and becomes nearly as active as \( \mu \)–I–II. Elimination of the bridge in E333A \( \mu \)–II has a more drastic effect on enzyme activity, especially at low Ca\textsuperscript{2+} concentrations. Domain II Ca\textsuperscript{2+} binding appears essential, because Ca\textsuperscript{2+}-coordinating side-chain mutants E392R and D333A have severely impaired \( \mu \)–II activation and activity. The introduction of mutations into the whole heterodimeric enzyme that eliminate the salt bridge or Ca\textsuperscript{2+} binding to domain II produce similar phenotypes, suggesting that the protease core Ca\textsuperscript{2+} switch is crucial and cannot be overridden by Ca\textsuperscript{2+} binding to other domains.

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The abbreviations used are: PEF, penta EF-hand; DSC, differential scanning calorimetry; HSQC, heteronuclear single quantum coherence; IWF, intrinsic tryptophan fluorescence; SLY-MCA, succinyl-leucine-tyrosine-aminomethyl coumarin; MES, 4-morpholinolisethanesulfonic acid; wt, wild type; r.m.s.d., root mean square deviation.

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overall mechanism for realignment of the active site residues. This activation mechanism is central and fundamental to the calpains.

**EXPERIMENTAL PROCEDURES**

Cloning, Expression, and Purification of μ/m-minicalpain Hybrids and μ-II Mutants—The reciprocal μ/m minicalpain hybrid constructs, μI–mII and mI–μII, were generated by swapping μI or mII coding segments into the corresponding region of the previously described pET24d mI–II or μI–II constructs, both of which have a C-terminal His6 tag (22, 23). The AgeI unique restriction site, which occurs naturally in μ-calpain at the DI–DII boundary (μ–TTGGG25), was used as the swap site. An AgeI restriction site was engineered by Kunkel mutagenesis into the corresponding location within mI–II without alteration of its amino acid sequence using the primer ctcggcaatgccaccggtgaagtcttc–a (24). Swapping NcoI-AgeI fragments produced the desired mI–μII and μI–mII hybrids. Primers used to generate amino acid replacements were introduced in the D106A, NdeI agctccctggcatatggccgttcggstitution. In each case a diagnostic restriction site (underlined) was above after views are shown separately

C I and II. **FIG. 1. Schematic outline of Ca2+–induced conformational changes.** The positions of key residues and peptide backbone before and after Ca2+–induced conformational changes are signified by dotted and solid lines, respectively. The movement of specific residues is indicated by red arrows. Domains I and II are represented by blue and cyan, respectively. A, calcium site 1 in domain I. B, the double salt bridge linking domains I and II. C, calcium site 2 in domain II. Because of the complexity of the structural changes whereby two loops move to form site 2, the before and after views are shown separately above and below the thick arrow. D, retraction of the tryptophan wedge from the active site cleft.

(Cortec DNA Services Laboratory, Inc.) to confirm their identity and integrity. Expression in Escherichia coli, purification, and storage of the above constructs and their mutants followed the previously described protocols (22).

**Activity and Intrinsic Tryptophan Fluorescence Assays—**The steady-state kinetics of mutants and μ/m minicalpain hybrids were measured under similar conditions to those originally described for μ–I–II using the peptide, succinyl-leucine-tyrosine-aminomethyl coumarin (SLYMCA, Sigma), as substrate (22). These conditions were also maintained for the CaCl2 activity titrations, which involved increasing CaCl2 con-
**Ca$^{2+}$ Switch Mechanism in Calpain**

**RESULTS**

**Validation of Mutants**—In this study, the Ca$^{2+}$-dependent mechanism by which the active site of calpain is aligned for catalysis was investigated using domain swaps and site-directed mutagenesis (Fig. 2) (22). Where targeted amino acid changes interrupt or alter a specific step in the process, it is essential to establish that the change in activity is due to the loss of a functional group and not due to the mis-folding of the mini-calpain. In general, mis-folded proteins are either poorly produced in E. coli or accumulate in inclusion bodies. All mutants and swaps used here expressed well in E. coli, remained soluble, and gave final yields of >10 mg/4 liters of culture that are comparable to those obtained with μI–II and μI–II. Another indication of correct folding is that the chromatography profiles of mutant and wild-type proteins were comparable (not shown). All the minicalpains produced discrete, sharp peaks that eluted at similar locations in the profiles.

To further validate the mutants, their thermal denaturation profiles were measured using DSC. Each minicalpain, mutant and wild type, showed a single thermal transition (Fig. 3A). The values ranged from 47 °C (wild type) to 44 °C (D106A). The latter mutant was the one for which an x-ray structure was determined, which revealed no significant differences in the protein fold. With the thermal transitions for the other mutants falling in between these flanking values, it can be safely assumed that none of them was structurally compromised. Limited proteolysis with chymotrypsin (Fig. 3B) and trypsin (not shown) confirmed this. The digestion products were essentially the same for each minicalpain after 80 or 320 min, the only difference being a slightly faster rate of breakdown for some mutants like E302R and W298A. The latter and E333A were the subject of NMR analysis by comparison of its HSQC spectra with that of the wild-type μI–II (not shown), which revealed no significant perturbations in the fold of the mutant.

**Experimental Strategy**—In conjunction with the domain swaps and site-directed mutagenesis, we have used two specific biochemical features of the calpain protease core to elucidate its mechanism of activation by Ca$^{2+}$. One is the large change in IWF that accompanies the Ca$^{2+}$-driven alignment of the active site for catalysis (22). We provide evidence below that this change is, as previously suspected, largely due to the movement of Trp298. The other is the Ca$^{2+}$-dependent proteolytic activity of the core as measured by SLY-MCA hydrolysis. The gain in enzyme activity for μI–II is biphasic. The first phase is sigmoidal. It reflects the cooperative binding of the two Ca$^{2+}$ and parallels the IWF change. The second phase is a gradual gain in activity at unphysiologically high Ca$^{2+}$ levels, which we attribute to general stabilization of the bilobal core. The core lacks the support of the other domains it would have in the whole enzyme. What readily distinguishes the m- and μ-mini-calpains as enzymes is the greatly reduced activity of mI–II due to the collapse of a key helix in domain I leading to a rearrangement of hydrophobic core residues (23).

**Minicalpain Domain Swaps Demonstrate the Order of Ca$^{2+}$ Binding**—The increase in IWF during Ca$^{2+}$ titration was measured for four minicalpains, native μI–II and mI–II, and the reciprocal domain swaps, mI–μI and μI–mI. All four gave characteristic sigmoidal profiles that fitted well to the Hill equation (Fig. 4A and Table II). Ca$^{2+}$ binding was cooperative between the two domains as indicated both by the upward curvature of the Scatchard plots (Fig. 4A, inset) and by the Hill coefficients of ~2. Ca$^{2+}$ concentrations for half-maximal change ([Ca$^{2+}$]) were ~27 and 190 μM for μI–II and mI–II, respectively (Fig. 4A and Table II). These values are remarkably close to the Ca$^{2+}$ requirement for activation of the intact μ- (5–50 μM) and m-calpains (200–1000 μM). The Ca$^{2+}$ requirements for the mI–μIII and μI–mIII swaps were 33 and 104 μM, respectively (Fig. 4A and Table II), intermediate between those of μI–II and mI–II. Even more informative was the shape of the sigmoidal IWF profiles. The μI-containing constructs, μI–II and mI–mI, produced very similar profiles that saturated at ~200 μM, whereas the μII-containing constructs, mI–II and μI–mI, both reached a plateau at ~1 mM. Thus, it appears domain II controls the end point of the titration and binds Ca$^{2+}$ second. The initial part of the transition was very similar for the μI-containing constructs, μI–II and mI–μI, with both proteins beginning their change at <10 μM Ca$^{2+}$. We, therefore, reasoned that DI from either isozyme has high Ca$^{2+}$ affinity and binds Ca$^{2+}$ first, with μI being a slightly better binder than mI.

We further demonstrated the functionality and hybrid nature of the domain swaps by testing their enzyme activity. We have previously shown that μI–II readily hydrolyzes the calpain substrate SLY-MCA (22). In contrast, mI–II fails to cleave SLY-MCA because of an intrinsic inactivation mechanism triggered by the collapse of a key α-helix at Gly203 in domain I (23). This occurs in the Ca$^{2+}$-loaded state and leads to the pruchusion of Trp108 into the active site cleft. Consistent with this, the

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**Table I**

| Crystalllographic data | Total | Outer shell |
|------------------------|-------|-------------|
| Resolution range (Å)   | 50.00–1.85 | 1.92–1.85   |
| Measured reflections   | 304,165 |             |
| Unique reflections      | 26,600  | 2,212       |
| Completeness (%)        | 97.1    | 86.9        |
| l/σl                   | 23.4    | 4.0         |
| Rmerge / Rcoeff (%)     | 7.8 (6.3) | 41.2 (31.5) |
| Refined structural model |      |             |
| Free reflections (5% of unique) | 1.283 |             |
| Rfree (%)               | 23.9    |             |
| Rfree (%)               | 25.9    |             |
| N. of protein/solvent/Ca$^{2+}$ atoms | 2,537/101 |             |
| Bond angle r.m.s.d. (°) | 1.194  |             |
| Bond length r.m.s.d. (Å) | 0.005  |             |
| Average Bfactor (Å$^2$) | 30.3   |             |

* Rmerge = Σ[I1 – (I2)]/ΣI2, where I1 represents individual measurements for any one reflection, and I2 is the average intensity of the symmetry equivalent reflections.

Rcoeff = Σ[I1 – (I2)]/ΣI2.

Rmerge = Σ[(Fobs – Fcalc)]/ΣFobs, where Fobs and Fcalc are observed and calculated structure factor amplitudes, respectively.

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**Crystallization and Structure Determination**—Crystallization of D106A/C115S μI–II in the presence of Ca$^{2+}$ was done by the hanging drop, vapor diffusion method under slightly different conditions from those established for wild-type μI–II, and produced crystals of a new space group. The well solution contained 1.5 M NaCl, 0.1 M MES (pH 6.5), and 10 mM CaCl2. The drop size was less than 5 µl and contained an equal volume of well solution and protein solution (12.5 mg/ml). Crystals were cryo-protected by six serial soakings (each for up to 5 min) in stabilization solutions containing increasing 5–30% (v/v) glycerol. Diffraction data were collected at the Cornell High Energy Synchrotron Source beamline X10, which was equipped with a Quantum-4 charge-coupled device detector (Area Detector Systems Corp.) and a liquid nitrogen cryo unit (Oxford Cryosystems). The diffraction data were processed as previously described for μI–II (22). The space group was P212121, with one molecule per asymmetric unit (Table I). The structure solution was determined by molecular replacement using AMoRe, and the structure of the Ca$^{2+}$-bound μI–II (PDB accession code 1KXR) as a model. Refinement and analysis of the structure were performed using the software described for μI–II (22). The ribbon diagrams were generated using Molscript (26). The electron density diagram was made using Xfit, and all structure figures were rendered in RASTER3D (27, 28).
activity of the mI-containing swap, mI–II, was barely detectable with a $k_{cat}$ of $3.6 \pm 0.9 \times 10^{-2} \text{s}^{-1}$, and a $K_m$ of $0.46 \pm 0.01 \text{mM}$ (Fig. 4B and Table II). In contrast, the mII-containing swap, mII–mII, where there is an alanine in place of Gly203, was quite active. Its $k_{cat}$ ($2.3 \pm 0.3 \times 10^{-2} \text{s}^{-1}$) was about half that of mI–II, and its $K_m$ was slightly higher ($0.403 \pm 0.009 \text{mM}$ versus $0.3 \text{mM}$). As seen with mI–II, the activity of mII–mII (but not that of mI–mII) was augmented at higher ionic strength due to general stabilization of the core.

We have also modeled the swaps using the Ca$^{2+}$-bound mI–II and mII–II structures (22, 23). The domain interface is highly conserved between the isoforms (not shown), and there are no obvious structural impediments to the hybrids undergoing activation by the same mechanism that brings the two domains together in mI–II. Several inferences can be made from the domain swap analysis. Regardless of isoform, the domain I site has a very similar high affinity for Ca$^{2+}$, being occupied prior to domain II. In contrast, the affinity of domain II is isoform-specific being the principal determinant of Ca$^{2+}$ requirement within the protease core.

Trp$^{298}$ Contributes Most to the Intrinsic Trytophan Fluorescence Change of mI–II—Ca$^{2+}$ loading results in a very substantial IWF increase (~37% in mI–II and ~28% in mII–II) that shows the cooperativity of the process and makes the conformational change easy to follow. The prime candidate for the major contributor to the IWF increase is Trp$^{298}$ (22). Its indole ring moves from the wedge-like position in between domains, where it is exposed to solvent on both sides, into a Ca$^{2+}$-induced hydrophobic pocket that fully buries one of its sides (Fig. 1). Because the Trp$^{298}$ residue remains surface-accessible, rather than contributing to a hydrophobic core, its mutagenesis to alanine is structurally feasible. Its position above the domain II catalytic triad residues (His$^{272}$ and Asn$^{296}$) matches to alanine is structurally feasible. It is involved in major stabilizing interactions in the Ca$^{2+}$-free structure and is more solvent-exposed than Glu$^{185}$, it was chosen for mutation to A (D106A).

The IWF profile of D106A mI–II during Ca$^{2+}$ titration was significantly different from that of wild-type mI–II and produced only a 5% total change in IWF (Fig. 5A and B), suggesting that its replacement might negatively affect DI folding and stability. In contrast, Asp$^{106}$ is more peripheral, being part of the flexible loop (residues 99–106) that closes in to provide the other protein coordinations to the Ca$^{2+}$ site. Because its side chain is not involved in major stabilizing interactions in the Ca$^{2+}$-free structure and is more solvent-exposed than Glu$^{185}$, it was chosen for mutation to A (D106A).

The calcium requirement for this transition was much greater than for the wild type (IC$_{50}$ of $15 \mu$M). The poor fit to the Hill equation, as indicated by a Hill coefficient of $<1.3$ and a much flatter Scatchard profile, suggested that the domain I Ca$^{2+}$ binding site was abolished in this mutant (Fig. 5B) along with the natural cooperativity between the two sites. We also followed the activity of these minicalpains during Ca$^{2+}$ titrations by monitoring the hydrolysis of SLY-MCA. The initial Ca$^{2+}$-dependent sigmoidal increase in wild-type mI–II

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**Fig. 2.** Calpain constructs, mutations, and E302R modeling. A, the m-calpain large subunit (m-80 kDa), truncated small subunit (ss 1–21 kDa) and minicalpain constructs are shown as colored bar diagrams with a C-terminal His tag ($H_6$). Domain boundaries are indicated by amino acid numbers below the bars, and mutations are shown by arrows above the particular construct. The catalytic triad residues are numbered on the m-80 kDa large subunit. The colors of construct abbreviations (left) and mutants (above) are kept the same throughout the manuscript within the graphs and structure diagrams. PEF, penta EF-hand. B, stereoview diagram of a portion of Ca$^{2+}$-bound mI–II minicalpain showing the residues underlying the activation mechanism and positions (colored) of mutated residues. C, E302R substitution based on a model of the DI Ca$^{2+}$ site in the active configuration.
activity (Fig. 4A, blue trace), which correlated with the increase in IWF and was complete at 200 μM Ca²⁺, represents enzyme activation (22). This was followed by a nonspecific, gradual increase (hyperbolic) in activity brought about by divergent cations (and to a lesser extent by monovalent cations), which we have argued represents structural stabilization of the enzyme (22). (Within the heterodimeric calpains the C₂-like domain III may fulfill this role of stabilizing the core.) At low Ca²⁺ levels (<200 μM), the D106A mutant of μI-II gave a hyperbolic increase in activity instead of the wild-type sigmoidal change, suggesting that only one Ca²⁺ site was functional (Fig. 6A, yellow trace). Despite this and the difference in IWF profiles, D106A was a surprisingly efficient enzyme. Indeed, the SLY-MCA hydrolysis kinetics of both wild type and D106A were comparable over the low mM Ca²⁺ range (Fig. 6A).

D106A Crystal Structure Reveals an Empty DI Ca²⁺ Site—To check for structural changes induced by the D106A mutation and to be sure that it completely abolished Ca²⁺ binding at the DI site we solved the D106A/C115S μI-II structure to 1.9-Å resolution. We established new conditions for the crystallization of μI-II in the presence of Ca²⁺, which, in comparison to the original conditions (22), produced more stable crystals with higher symmetry that diffracted at higher resolution (Table I). After the first round of refinement of the Ca²⁺-bound μI-II model, which was used to obtain the molecular replacement solution for D106A μI-II, the 2Fo − Fc map indicated that density corresponding to the Asp¹⁰⁶ side chain was absent beyond the CB, as expected for the D106A substitution (not shown). In addition, the density corresponding to the Ca²⁺ ion in domain I of μI-II was not matched in the new map. Upon Ca²⁺ removal from the model, the following round of refinement confirmed the absence of a Ca²⁺ ion at this position, which was instead filled by a water molecule (wat1, Fig. 7, B and C). The second water molecule that coordinates the Ca²⁺ in μI-II (wat2, Fig. 7B) is absent in the D106A mutant structure, suggesting that Ca²⁺ likely does not bind at this site even in the presence of 10 mM CaCl₂ used during crystallization. Higher resolution data (~1.5 Å) might permit refinement of the occupancy at this site, which is further hampered because of the weak contribution of Ca²⁺ to the electron density (20 electrons).

With the exception of these changes, the Ca²⁺-bound structure of D106A was essentially identical to μI-II (Fig. 7A), even within the loop that contains D106A, meaning that this was in the “closed” Ca²⁺-coordinating position despite the absence of Ca²⁺ in site 1 (Fig. 7B). The relative orientation of domains and realignment of active site residues was preserved. In addition, all critical interactions directly involved in the activation mechanism were maintained, including the Arg¹⁰⁴-Glu³³³ salt bridge, the occupied Ca²⁺ site in DI, and the Ca²⁺-induced Trp²⁹⁸ pocket (Fig. 7A). The D106A μI-II structure shows that at unphysiologically high Ca²⁺ levels used in vitro, Ca²⁺ binding to domain I can be circumvented in the mutant. Activation of D106A μI-II likely follows a slightly different molecular mechanism. We suggest that the loop that harbors Arg¹⁰⁴ could move close to the Glu³³³ side chain, because it is free of electrostatic clashes that would otherwise be caused by the Asp¹⁰⁶ side chain in the absence of Ca²⁺. In this process Arg¹⁰⁴ would already be repositioned into the observed Ca²⁺-bound orientation, and would in turn facilitate the rotation and side-chain removal of Glu³³³ from the domain II Ca²⁺ site without having
to overcome the energetic barrier imposed by forming the domain I Ca²⁺ site (Fig. 1).

The Arg¹⁰⁴-Glu³³³ Salt Bridge Is More Important for Activation than Ca²⁺ Site 1—The double salt bridge, Arg¹⁰⁴-Glu³³³, is the direct physical link between the Ca²⁺ sites in domains I and II and, therefore, the underlying structural basis for cooperativity in the core (Fig. 1). To test this hypothesis, we abolished this interaction through E333A mutation (Fig. 2). This mutation radically changed the IWF profile during Ca²⁺ titrations (Fig. 5). There was no increase in IWF until after 100 μM CaCl₂, which is consistent with the inability of Ca²⁺ site I binding to influence the transition, site I being the first to bind Ca²⁺ (Fig. 5A). The data from the beginning of the fluorescence increase (Fig. 5B, orange trace, [Ca²⁺]_0 ≈ 300 μM) did not fit the Hill equation, despite a Hill coefficient of 1.8. They generated the most severely flattened Scatchard plot of the series, indicating a major disruption in cooperativity (Fig. 5B, inset). Enzyme activity was also severely impaired at low Ca²⁺ concentrations. It became detectable at 300–400 μM CaCl₂ (Fig. 6A), but the initial activation phase was stretched out to ~10 mM CaCl₂ (not shown) compared with ~200 μM for µI–II, and it saturated at a lower value (Fig. 6A).

Domain II Ca²⁺ Binding Defines the Next Step in the Activation Hierarchy of µI–II—Having probed the domain I Ca²⁺ site and the double salt bridge between domains I and II, we next looked at the domain II Ca²⁺ site. A critical side-chain rearrangement must be made by residue Glu³⁰² to form this second Ca²⁺ site (22). Glu³⁰² exchanges position with Val¹⁰¹ to provide double coordinations to Ca²⁺, while creating room for a short anti-parallel β-sheet (Fig. 1). This sheet, along with the Val¹⁰¹ side chain, lines the periphery of the Trp²⁹⁸-binding pocket together with the Ile²⁶² and Val²⁶⁹ side chains. We considered that E302R might generate a Ca²⁺-independent enzyme, because the arginine side chain can be modeled with its guanidino group within the Ca²⁺ binding position, where it could interact electrostatically with the rest of the coordination complex (Fig. 2C). This thought was partly driven by the three-dimensional alignment model of the Ca²⁺-bound calpain 7 DI–II (not shown), which contains an Arg at this position and is believed to be Ca²⁺-independent (2). When Arg replaced Glu³⁰², the mutation abolished enzyme activity even at elevated divalent cation levels. This was despite an associated 15% increase in IWF (Fig. 5A, green trace) that started after ~20 μM CaCl₂ and fitted poorly to the Hill equation ([(Ca²⁺)]₀.⁵ ≈ 102 μM, Hill coefficient ≈ 1.3, Fig. 5B). E302R presumably blocked formation of a functional domain II Ca²⁺ site in µI–II.

**Fig. 5. IWF analysis of activation mechanism mutants.** The data for IWF during Ca²⁺ titrations of µI–II mutants are shown in the raw form (A) or normalized (B) with the corresponding Scatchard plots shown in the B inset. Minicalpain (~0.4 μM) was titrated with CaCl₂ as described under Experimental Procedures. Colors for the wild-type µI–II (blue), W298A (gray), D106A (yellow), E302R (green), D331A (pink), and E333A (orange) are as seen in Fig. 2.

**Table II**

| (Ca²⁺)_0³ | n_H | k_cat | K_M | k_cat/K_M |
|-----------|-----|-------|-----|-----------|
| µI–II     | 26.5±1.0 | 1.8 | 42.7±4.7 | 335±6 | 130 |
| µI–III    | 104±33  | 1.4 | 23.0±0.3 | 403±9 | 57  |
| µI–II     | 32.9±3.4| 1.7 | 3.6±0.9  | 460±10 | 8   |
| µI–III    | 190±4  | 1.7 | ND¹ | ND   | Inactive |
| D106A µI–II | 144±21 | 1.3 | ND | ND | Inactive |
| W298A µI–II | 15±3  | 1.8 | ND | ND | Inactive |
| E302R µI–II | 119±3 | 1.4 | 1.4±0.5 | 318±20 | 44 |
| D331A µI–II | 103±6 | 1.3 | (33.1±2.5)¹ | (304±9)¹ | (109)¹ |
| E333A µI–II | 292±3 | 1.8 | ND | ND | Inactive |
| m80k/21k  | 353±9  | 5.6 | 3,248±60 | 211±9 | 65,000 |
| R94G m80k/21k | 1,485±63 | 1.9 | 2,246±104 | 189±6 | 11,860 |
| ED320/321AA | 8,352±641 | 1.3 | 1,341±169 | 131±12 | 10,182 |

¹ (Ca²⁺)_0 is the half-maximal Ca²⁺ requirement obtained during intrinsic tryptophan fluorescence titrations.  
² n_H is the Hill coefficient.  
³ k_cat/K_M refers to the nanomoles of MCA released per second per micromole of enzyme.  
⁴ k_cat/K_M is the catalytic efficiency of MCA release.  
⁵ ND, not determined.  
⁶ The parameters obtained at 20 mM CaCl₂.
and prevented the formation of the Trp298 pocket, suggesting that proper site 2 assembly is essential for activation.

We therefore made a less disruptive substitution of a Ca\(^{2+}\)-coordinating residue in DII, namely the D331A mutation. D331 provides one-side-chain coordination to Ca\(^{2+}\) as well as a stabilizing coordination to the water molecule that in turn coordinates Ca\(^{2+}\) (Fig. 2B) (22). This side chain is somewhat secondary to the activation mechanism, not being involved directly in critical inter-stage transitions such as Glu333 or Glu302 repositioning. Nevertheless, this mutation impaired the activation mechanism more so than the E333A salt bridge mutation as observed with any of the PEF mutants or PEF DIV swaps, requiring the active site residues, the cooperative link (dashed lines), and Ca\(^{2+}\) ion positions. Ca\(^{2+}\) ions are shown as transparent pink (µI–II) and yellow (D106A µI–II) spheres. B, stereoview of DI site overlap indicating the D106A substitution (yellow) within loop 98–107, which contains three Ca\(^{2+}\) coordinating residues. The positions of the three water molecules (wat1–3, light green) in D106A structure and wat4 (pink) in the wild-type structure are shown with respect to the missing Ca\(^{2+}\) (transparent pink) and Arg104 side chain. C, stereoview of the 2Fo – Fc, electron density map (violet) at 1.9-Å resolution contoured at 1σ level for D106A µI–II DI site. Carbon atoms are colored blue, and the water molecules are light green.

enzyme, we chose to abolish these critical stages within the m-calpain heterodimer. R94G was used to mimic the effect of E333A in eliminating the salt bridge, and the E320A/D321A double mutation was used in the manner of D331A to retard activation, respectively compared with E320A/D321A mutants during the activation stage. R94G and E320A/D321A required −1.5 mM and −8 mM CaCl\(_2\) for half-maximal activation, respectively, compared with −350 μM for the wild type (Fig. 6B). These extremely high Ca\(^{2+}\) requirements were never achieved using any single site mutation within the DIV or DVI PEF domains (30). The most severe single EF-hand mutation only increased the CaCl\(_2\) concentration for half-maximal activation to 0.83 mM. In addition, these enzymes were weaker, both being −20% as active compared with wild type at 20 and 60 mM CaCl\(_2\), respectively (Table II). A lower activity was not observed with any of the PEF mutants or PEF DIV swaps, which essentially have wild-type activities but with a different

Effects of Activation Mechanism Mutations on m-calpain Heterodimer—Based on these minicalpain mutation studies, the double salt bridge Arg104,Glu333 formation and the proper assembly of the DII Ca\(^{2+}\) site are the most critical stages of the activation mechanism, the latter being significantly more important. To validate this conclusion in the context of the whole

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Ca$^{2+}$ requirement (30, 31). These results suggest that the activation mechanism observed within the isolated protease core can be confidently extended to the activation of the core within the context of the heterodimer. In this respect DII Ca$^{2+}$ site assembly is the ultimate rate-limiting event during the heterodimer activation, being strongly facilitated by the Arg$^{104}$-Glu$^{333}$ cooperative interaction.

**DISCUSSION**

The presence of 10-12 Ca$^{2+}$ binding sites throughout calpain complicates the issue of cooperativity in the whole enzyme, despite the structural information gathered thus far (22, 30). In contrast, the protease core deals only with one pair of sites that act cooperatively to realign the active site cleft for catalysis. A comparison of the crystal structures of apo m-calpain and Ca$^{2+}$-bound μ-I–II reveals the net changes that are required for activation through the realignment of the active site (21, 22). From these snapshots we previously surmised that the two non-EF-hand Ca$^{2+}$ sites were physically and mechanistically linked through the double salt bridge, but we had no biochemical evidence for this or for the order of progression of Ca$^{2+}$ binding (22). Elucidation of the activation mechanism is provided here through biochemical analysis of μ and μI–II domain swaps and μI–II mutants of select residues involved at sequential steps of the mechanism.

Evidence for the order of Ca$^{2+}$ binding came initially from the domain swaps and their IWF change during Ca$^{2+}$ titration, which indicated that domain I binding determines the onset of the sigmoidal IWF profile, and domain II binding controls its saturation. We had previously shown that the 4- to 5-fold difference in Ca$^{2+}$ requirement of the μ- and m-calpain isoforms is faithfully reflected in the Ca$^{2+}$ requirement for activation within the protease core, being complete at ~200 μM Ca$^{2+}$ for μI–II and at ~1 mM Ca$^{2+}$ for μ-II (23). We now show that saturation is largely determined by domain II, because both μII-containing minicalpain complex their titration at ~200 μM Ca$^{2+}$ whereas the μI-containing minicalpain do not reach saturation until ~1 mM Ca$^{2+}$. Thus, domain II is involved in the later stage of the active site assembly, which includes the removal of Trp$^{208}$ from its wedge-like position in the active site cleft. This cannot occur until site 2 is formed. Although Trp$^{208}$ is one of 11 Trp residues in μI–II, its mutation to Ala reduced the IWF change by ~75%. (The other 10 Trp residues are found in overlapping positions in the apo-μ and holo-μ protease cores.) This mutation helped establish the order of Ca$^{2+}$ binding. W298A abolished the final stage of the sigmoidal IWF transition, emphasizing its involvement during the later stages of the activation mechanism. This Trp is present in all papain-like cysteine proteases, but only in calpains does it function as a removable wedge and undergo a radical change in environment with activation (32).

The fact that part of site 1 is already pre-assembled is suggestive of a role in the early stages of activation, but direct evidence that domain I binding determines the onset of the sigmoidal IWF profile came from the salt bridge knock-out mutation E333A. This mutation essentially uncoupled Ca$^{2+}$ binding to site 1 from the activation process. All cooperativity was lost, and the IWF transition did not begin until after 100 μM Ca$^{2+}$ was reached. Clearly, what should have been an early boost from site 1 binding was unable to initiate the cooperative process, because the distal part of the salt bridge was missing and unable to transmit the Ca$^{2+}$ signal. E333A mutation also resulted in ~10-fold increase in the Ca$^{2+}$ requirement for activation, suggesting that domain II site formation is greatly facilitated by the cooperative link, which helps remove the Glu$^{333}$ side chain from the Ca$^{2+}$ ion position.

Support for the cooperativity between the two protease-core Ca$^{2+}$ sites came from disruption of either end of the Arg$^{104}$-Glu$^{333}$ double salt bridge, through E333A substitution in μI–II and R94G in m-calpain. Sequence alignments revealed that about two thirds of the calpain isoforms have conserved the Arg-Glu salt bridge, further supporting the role for this ancestral cooperative link. In addition, other key residues (identified in Fig. 2B) are >95% conserved among all large subunit homologues, perhaps indicating the mechanism’s importance in conveying the Ca$^{2+}$ signal to the active site within these isoforms, and suggesting an ancestral common origin for these calpains. The R94G mutation within the context of the heterodimeric m-calpain severely impaired activation and diminished activity (33). The equivalent mutation (R118G) occurs in the muscle-specific calpain isoform, p94, in some patients with limb girdle muscular dystrophy 2A (13). Loss of function of this enzyme is responsible for the disease (35). R118G is one of many inactivating mutations catalogued in p94 but one of very few, which can now be explained mechanistically in light of our corresponding mutation in m-calpain. From this study we conclude that p94 R118G activity is impaired due to loss of site cooperativity within its protease core.

A completely unexpected finding of the mutagenesis experiment was the resilience of the D106A mutation, which was designed to knock out the domain I Ca$^{2+}$ binding site. Although the crystal structure showed that Ca$^{2+}$ was missing from site 1, the movable loop containing D106A had in fact closed up to near the normal position but with water in place of Ca$^{2+}$. Passive closure of this loop in the absence of Ca$^{2+}$ would of course permit the double salt bridge to form and facilitate Ca$^{2+}$ binding to site 2. Also, under the high Ca$^{2+}$ (10 mM) crystallization conditions, occupancy of site 2 might form the salt bridge from the reverse direction. Formation of the salt bridge would clearly facilitate the closing up of the movable site 1 loop toward Glu$^{185}$ and, unlike Asp$^{106}$, Ala$^{106}$ would not cause charge repulsion in the absence of Ca$^{2+}$. However, in the wild type, domain I site confers cooperativity to the protease core and directly modulates the activation mechanism. We conclude that the cooperative link is more important than the domain I site, because it forms despite the D106A mutation and it more directly facilitates Ca$^{2+}$ binding to the rate determining domain II site.

It appears that mutations introduced along the mechanism from the domain I site, into the cooperative link, and further into the DII site have increasingly dramatic effects on activation, suggesting a similar hierarchy of significance for these stages. In this regard, the domain II site mutations within the m-calpain heterodimer, E320A/D321A, had more severe effects on activation and activity than mutations of any single EF-hand motif within the PEF domains IV and VI (30). Loss of Ca$^{2+}$ binding in domain II was more disruptive than losing the double salt bridge through R94G substitution. This result extends our model of heterodimer activation, which begins with Ca$^{2+}$ binding at both PEF domains and the concomitant release of the N-terminal large subunit anchor from the small subunit (22). These initial events release some of the tension on the protease core. The involvement of domain III in the protease core stabilization is postulated indirectly from the nonspecific stabilization of the core by divalent cations (23) and directly through mutations at the domain II-domain III interface (36). However, the putative Ca$^{2+}$ binding ability of this C2-like domain should not be excluded as a specific contributor to the Ca$^{2+}$-induced release of tension on the core (37). When freed of tension, the protease core should realign through our proposed mechanism, which makes domain II occupancy the last and crucial rate-limiting step of heterodimer activation, and by analogy that of most calpain isoforms. Although additional work is needed to address the activation of the more
complex calpains, our mechanism might completely describe the activation of some of the least complex ones, including nCL-2 (2) and the naturally occurring protease cores (akin to μ1–II) generated through calpain autolysis. In stark contrast to the initial concept that PEF domains were central to calpain activation by Ca\(^{2+}\) (2, 18, 21), it appears that they are later additions to further control activation by Ca\(^{2+}\) signaling. Calpain activation originates in the core and was the key step in evolving a Ca\(^{2+}\) dependent cysteine protease from a papain-like progenitor.

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