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m-Calpain activation *in vitro* does not require autolysis or subunit dissociation

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ABSTRACT

Calpains are Ca^{2+}-dependent, intracellular cysteine proteases involved in many physiological functions. How calpains are activated in the cell is unknown because the average intracellular concentration of Ca^{2+} is orders of magnitude lower than that needed for half-maximal activation of the enzyme *in vitro*. Two of the proposed mechanisms by which calpains can overcome this Ca^{2+} concentration differential are autoproteolysis (autolysis) and subunit dissociation, both of which could release constraints on the core by breaking the link between the anchor helix and the small subunit to allow the active site to form. By measuring the rate of autolysis at different sites in calpain, we show that while the anchor helix is one of the first targets to be cut, this occurs in the same time-frame as several potentially inactivating cleavages in Domain III. Thus autolytic activation would overlap with inactivation. We also show that the small subunit does not dissociate from the large subunit, but is proteolyzed to a 40–45 k heterodimer of Domains IV and VI. It is likely that this autolysis-generated heterodimer has previously been misidentified as the small subunit homodimer produced by subunit dissociation. We propose a model for m-calpain activation that does not involve either autolysis or subunit dissociation.

1. Introduction

Calpains are a family of intracellular cysteine proteases that are found in both vertebrates and invertebrates. They catalyze the limited cleavage of protein substrates when activated by Ca^{2+}, essentially converting local cellular Ca^{2+} signals into a wide range of cellular responses, including cell motility, cell cycle regulation, transcriptional regulation, signal transduction, and apoptosis (reviewed in Ref.[1]). The direct or indirect involvement of calpains in many diseases, including but not limited to traumatic brain injury, Alzheimer's disease, myocardial ischaemic injury, limb-girdle muscular dystrophy type 2A, gastric cancer, and type 2 diabetes mellitus [2–8], emphasizes the importance of calpains' roles and functions.

μ- and m-Calpains, the two most studied calpain isoforms, are ubiquitously expressed and are heterodimers of an 80 k large subunit (Domains I–IV) and 28 k small subunit (Domains V–VI). Domains I and II, the "protease core", contain the catalytic triad residues: the active site cysteine in Domain I and the histidine/asparagine pair in Domain II. Domain III, the C2-like domain, has been suggested to play a role in regulating calpain activity via electrostatic interactions with other domains [9], and interactions with cell membranes by phospholipid binding [10]. The last domain in the large subunit, Domain IV, has five EF-hands. The first four EF-hands contain calcium binding sites while the fifth EF-hand, the one closest to the C terminus, is involved in heterodimerization of the large and small subunits of calpain [9,11–14]. Domain V, at the N terminus of the small subunit of calpain, is glycine-rich and may have an unordered structure. Domain VI of the small subunit is homologous to Domain IV of the large subunit and forms a tight interaction with Domain IV through the pairing of their penta-EF-hands; thus Domains IV and VI are often termed the PEF-domains. The crystal structure of the apo-form of m-calpain lacking Domain V [9,12] revealed that the catalytic triad is misaligned prior to activation. In the absence of Ca^{2+}, the anchor helix at the N terminus of Domain I of the large subunit interacts with Domain VI of the small subunit. Along with the penta-EF-hand heterodimerization between Domains IV and VI, the anchor helix effectively circularizes all six domains and helps to structurally restrain calpain in an inactive conformation.

In the presence of sufficient Ca^{2+}, relief of structural constraints on the protease core allows the rotation of these two domains relative to each other, resulting in realignment of the catalytic triad to form the catalytic cleft [15]. However, the resting intracellular Ca^{2+} concentration across the cytoplasm (~0.1 μM) is orders of magnitude lower than that required for half-maximal activity of either μ-calpain (~50 μM) or m-calpain (~500 μM) *in vitro*[1,16].

One frequently cited explanation for overcoming this Ca^{2+} barrier in the cell is autoproteolysis (autolysis) of the anchor helix (Fig. 1A), which is claimed to lower the Ca^{2+} concentration needed for activation [17–22]. Subunit dissociation, where the small subunit separates from the large subunit and forms small subunit homodimers (Fig. 1B), is another published and widely quoted mechanism that is thought to facilitate calpain activation [23–28]. In both cases, removal of either the anchor

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helix or the small subunit could in principle relieve structural constraints on the protease core, allowing the realignment of the core domains required for activation. Furthermore, researchers have recently reported that the two mechanisms are not mutually exclusive, claiming that autolysis induces subunit dissociation [29,30]. However, other groups have found that the small subunit does not dissociate [31,32]. Mutation of the small subunit EF-hands affects the Ca^{2+} requirement of the whole enzyme, which suggests that it remains bound to the large subunit during activation [11]. Recently, the Ca^{2+}-bound structure of active m-calpain [13,14] showed that the small subunit is present in the activated enzyme and that it makes new heterodimeric interactions after activation.

Given the disagreement in the literature about the contribution of autolysis and subunit dissociation to calpain activation, we have set out to investigate these two processes. Careful separation and analysis of end-stage calpain autolysis products showed that the PEF-domains remained together as heterodimers without any sign of dissociation or reorganization into homodimers. Mass spectrometric analysis of the early autolysis products showed that the anchor helix was extensively cleaved during the first minute of Ca^{2+} addition but numerous sites in Domain III were also cut during this initial phase of autolysis. Since cleavage of Domain III in m-calpain can release inactive protease core [33], and cuts here occur within the same time-frame as anchor helix cleavage, autolysis is unlikely to have evolved as a mechanism for calpain activation.

2. Materials and methods

2.1. m-Calpain protein purification and autolysis time course

Rat calpain 80 k large subunit and truncated 21 k small subunit were co-expressed and purified as previously described [34]. Purified m-calpain protein (70 μg) was diluted into 100 μL of Buffer A (50 mM Tris–HCl pH 7.6, and 10 mM β-mercaptoethanol) and autolysis was initiated by the addition of $CaCl_2$ to a final concentration of 1 mM. Aliquots (7 μg) were removed from the reaction at various time points (1, 2, 5, 10, 15, 20, 40, and 180 min) and mixed with 10 μL of SDS loading buffer + 25 mM EDTA to stop the reaction. Time point samples were then analyzed by 10% (w/v) SDS-PAGE. Autolysis samples prepared for mass spectrometry analysis were digested in 50 mM HEPES pH 7.4, and 10 mM dithiothreitol without any obvious difference in the pattern of cleavage products.

2.2. m-Calpain autolysis site determination by mass spectrometry

Time course samples were analyzed following modification of N-terminal peptides as previously described [35]. Briefly, aliquots containing ~1.2 nmol of calpain and its autodigestion products were dissolved in 500 μl 2 M guanidinium hydrochloride in 50 mM sodium phosphate pH 8.0. Trideuteroacetate-N-hydroxysuccinimide (AcD3-NHS, final concentration of 12.5 mM) was added to each sample to modify α- and ε-amines during a reaction for 2 h at 30 °C. Hydroxylamine (final concentration of 75 mM) and glycine (final concentration of 25 mM) were added and samples were incubated for 20 min at room temperature to reverse potential O-trideutero-acetylation on Ser, Thr or Tyr residues and quench non-reacted NHS-ester, respectively. Samples were desalted on NAP-5 columns (GE Healthcare Life Sciences) and recollected in 1 mL of 20 mM ammonium bicarbonate pH 8.0. Samples were boiled for 5 min, put on ice for 5 min and digested overnight at 37 °C with 2 μg trypsin (Promega Sequencing Grade Modified Trypsin). Without any pre-enrichment for N-terminal peptides, 100 μL of each sample was acidified with 2 μL formic acid and used for LC-MS/MS analysis on a Thermo LTQ-Orbitrap XL mass spectrometer (2 μL of each sample was injected) which was operated as described before [36]. Recorded MS/MS spectra were searched using a locally installed version of Mascot algorithm in the rat subsection of the Swiss-Prot database. The following search parameters were used: the protease was set to ArgC/P with up to one missed cleavage allowed, fixed trideutero-acetate modification of lysine residues, variable trideutero-acetate modification of peptide N-termini, variable oxidation of methionine residues to methionine-sulfoxide, a precursor mass tolerance of 10 ppm and a fragment mass tolerance of 0.5 Da. Two additional searches were performed to allow identification of neo-N-terminal peptides resulting from calpain autolysis. In these, Mascot's semi-ArgC/P protease settings were used and further peptide-centric databases holding all possible neo-N-termini from rat proteins were generated using the DBToolkit software as described previously [37,38]. Following Mascot database searches, peptides trideutero-acetylated at their α-amine were used to report calpain autolysis sites.

2.3. Size-exclusion chromatography

m-Calpain (0.37 mg) was autolyzed for more than 3 h in 0.5 mL of Buffer B (50 mM Tris–HCl pH 7.6, 10 mM β-mercaptoethanol, and 1 mM $CaCl_2$) to generate fully autolyzed calpain products. The digest was then run into a Superdex 75 10/300 GL column (S-75) that was equilibrated in Buffer B and coupled to the AKTA FPLC system (GE Healthcare Life Sciences). The column flow rate was set at 1 mL/min and 1 mL fractions were collected. Fractions corresponding to the peak seen at 280 nm were pooled, and 500 μL of this aliquot was concentrated using Vivaspin 500 (GE Healthcare Life Sciences) at 15,000 ×*g* before analysis by SDS-PAGE.

2.4. Phenyl sepharose chromatography

Fully autolyzed calpain (0.7 mg) was loaded onto a Phenyl FF (low sub) column 1 mL (GE Healthcare Life Sciences) in Buffer C (25 mM Tris–HCl pH 7.6, 600 mM NaCl, 10 mM β-mercaptoethanol, and 1 mM $CaCl_2$) at a flow rate of 1 mL/min. The column was washed with 2 column volumes of Buffer C and then eluted with a gradient from 0 to 100% Buffer D (25 mM Tris–HCl pH 7.6, 400 mM NaCl, 10 mM β-mercaptoethanol, and 2 mM EDTA) over 20 column volumes. Fractions (1 mL) containing protein peaks as detected by absorbance 280 nm were concentrated in Vivaspin 500 tubes and analyzed by SDS-PAGE.

2.5. Anion-exchange chromatography

Calpain (1.5 mg) was autolyzed in 2.0 mL of Buffer B before the fully autolyzed products were injected into a Mono Q 5/50 GL column (GE Healthcare Life Sciences). The column was washed with 10 column volumes of Buffer B before elution with a gradient from 0 to 100% of Buffer B + 500 mM NaCl at 1 mL/min. Fractions (1 mL) corresponding to protein peaks were collected, concentrated in Vivaspin 500 tubes, and analyzed by SDS-PAGE.

Pure truncated small subunit (DVI) was diluted into Buffer B, loaded, and eluted off the Mono Q column in an identical manner.

2.6. Trypsin digestion and identification of calpain peptides by MALDI-TOF mass spectrometry

After SDS-PAGE analysis, protein bands were carefully excised and destained in 50 mM ammonium bicarbonate in 50% acetonitrile. The samples were then reduced with DTT (dithiothreitol), alkylated with iodoacetamide, digested with 6 ng/μL trypsin (Promega Sequencing Grade Modified Trypsin) for 5 h at 37 °C, and recovered from the gel with three extractions of 1% formic acid in 2% acetonitrile.

Extracted peptides were mixed into the matrix solvent (5 mg/mL of α-cyano-4-hydroxycinnamic acid in 70% acetonitrile, 0.1% trifluoroacetic acid, and 10 mM di-ammonium citrate) in a 1:1 ratio. A spot of this final preparation at 1 pmol/μL was air dried and MALDI-TOF mass spectra were acquired and analyzed on a Voyager DePro (Applied Biosystems Corporation) using Data Explorer Version 5.1 software