FEBS Letters 581 (2007) 2894–2898

Calpastatin simultaneously binds four calpains with different kinetic constants

Rachel A. Hanna, Beatriz E. Garcia-Diaz[1], Peter L. Davies*

Department of Biochemistry, Queen's University, Kingston, Ontario, Canada K7L 3N6

Received 13 April 2007; revised 16 May 2007; accepted 16 May 2007

Available online 25 May 2007

Edited by Peter Brzezinski

Abstract **Calpastatin is the endogenous, specific protein inhibitor of the calcium-dependent protease, calpain. Using an active site knock-out m-calpain mutant we have studied the enzyme's calcium-dependent binding to calpastatin by surface plasmon resonance without the complication of proteolysis. Calpastatin was capable of simultaneously binding four molecules of calpain. Its four inhibitory domains (CAST1, 2, 3, and 4) were individually expressed in *Escherichia coli* and the kinetics of their interaction with calpain was separately compared. Their K_d values ranged from picomolar to nanomolar in the order CAST1 > 4 > 3 > 2. They have similar k_{on} values but the k_{off} values ranged over three orders of magnitude and can account for the differences in affinity.**

Keywords: Calpain; Protease; Calcium; Calpastatin; Inhibitor; Biosensor; Surface plasmon resonance

1. Introduction

Calpains are a family of cytosolic Ca^{2+}-dependent cysteine proteases that have been best characterized in animals, but are also found in plants, yeast and even bacteria [1]. They modulate many cellular processes by limited cleavage of specific protein substrates in response to calcium signaling. Some of their roles include cytoskeletal remodeling during cell fusion and cell motility, regulation of cell cycle progression, gene expression, and apoptotic cell death. Calpains have been implicated in many health problems including heart attack, stroke, limb girdle muscular dystrophy, cancer and type II diabetes.

The two most extensively studied mammalian isoforms, μ- and m-calpain (calpains 1 and 2, respectively), are ubiquitously and constitutively expressed. Both are heterodimers, each with an 80 k large subunit specific to the isoform, and a common 28 k subunit. Regulation of μ- and m-calpain in vivo is poorly understood. The process of calpain activation remains elusive, as the overall intracellular calcium levels in vivo are not sufficient to activate calpain in vitro [1–3]. There is speculation that protein binding partners and/or post-translational modification are required to lower the calcium requirement for activation. There is also speculation that calpains are activated at points in the cell by high local levels of calcium transported across the plasma membrane or released from an intracellular calcium store.

Calpastatin is the endogenous protein inhibitor of calpain [4]. To date it is the only inhibitor that is completely specific for calpain. It binds and inhibits calpain when calcium levels are high, but is released when calcium levels fall. Calpastatin contains five domains, four homologous domains (CAST1–4) of about 140 amino acids in length that are capable of inhibiting heterodimeric μ- and m-calpain and a unique N-terminal domain, termed domain L, with no inhibitory effect [5–10]. Calpastatin is well conserved with greater than 70% identity across mammalian species. It is encoded by a single gene that has no obvious homologues.

Calpastatin is an unstructured protein that has a complex interaction with calpain [5]. Each inhibitory domain contains three well-conserved subdomains, A, B and C [4]. Subdomains A and C interact at nM affinity with the two penta-EF-hand domains of calpain: A with domain IV on the large subunit of calpain, and C with domain VI of the small subunit [11,12]. Paradoxically, subdomain B, the subdomain responsible for inhibition, interacts weakly with calpain in the absence of its flanking A and C regions [7,13,14].

Previous work showed that all four inhibitory domains (CAST1–4) bind tightly to calpain, however accurate quantification of their affinity was not published [8,15]. In this study we have used surface plasmon resonance to measure the kinetic constants of individual CAST inhibitory domains and recombinant m-calpain. By using m-calpain with an active site mutation we have eliminated the complications of autoproteolysis and inhibitor digestion. In addition, the binding capacity of calpastatin for calpain was determined.

2. Materials and methods

2.1. Cloning of individual calpastatin inhibitory domains and calpastatin minus domain L (CAST1–4) fused to a C-terminal Ecoil peptide

The Ecoil:Kcoil *de novo* coiled–coil interaction was used to immobilize calpastatin on the surface of the Biacore CM5 chip so that the calpastatin was flexible and accessible to calpain (Fig. 1) [16]. The

*Corresponding author. Fax: +1 613 533 2497.
E-mail addresses: 9rah3@qlink.queensu.ca (R.A. Hanna), bgarciad@ucalgary.ca (B.E. Garcia-Diaz), daviesp@post.queensu.ca (P.L. Davies).

[1]Present address: Department of Biological Sciences, Biosciences Building, Room 418, University of Calgary, 2500 University Dr. NW, Calgary, AB, Canada T2N 1N4.

Abbreviations: CAST1, calpastatin inhibitory domain 1; CAST2, calpastatin inhibitory domain 2; CAST3, calpastatin inhibitory domain 3; CAST4, calpastatin inhibitory domain 4; CAST1–4, calpastatin polypeptide including domains 1–4

0014-5793/$32.00
doi:10.1016/j.febslet.2007.05.035

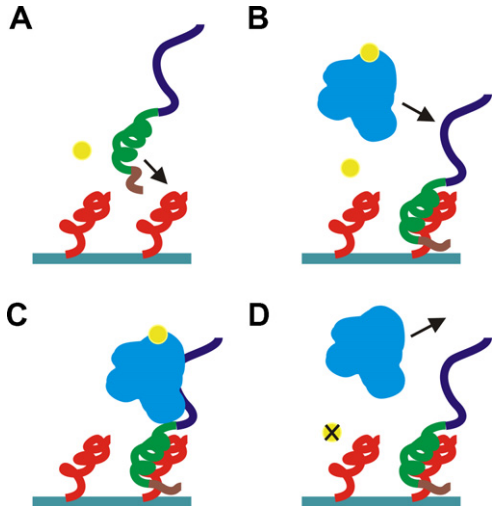

Fig. 1. Schematic outline of the Ecoil:Kcoil system used for kinetic analysis following the method of Tripet et al. [27]. (A) Preparation of the CM5 chip. Firstly the Kcoil peptide (red) is immobilized through its N-terminal cysteine to the chip surface via a thio-ether bond. Calpastatin inhibitory domain(s) (purple) followed by the Ecoil peptide (green) and a C-terminal His-tag (brown) is then injected and binds to the chip through the coiled–coil interaction. (B and C) Calpain (blue) is injected and binds to the immobilized calpastatin in the presence of calcium (yellow dots). (D) Calcium is removed by injecting buffer containing EDTA, signified by a cross on the yellow dot. Calpain dissociates from calpastatin in the absence of calcium and the calpastatin-bound chip is regenerated.

individual inhibitory domains were amplified by the polymerase chain reaction (PCR) from a clone of rat calpastatin cDNA lacking domain L (CAST1–4) supplied by Dr. Simon Arthur using the following primers: 5′CAST1 ATTCCGCTCG AGAAGCTTCC TAGGCGAACT TTCCCCAGTA GACTT, 3′CAST1 TTCAGATGAC CCCTTTTAAG TGGATCCTTC GAAGAGCTCG CCTTA, 5′CAST2 AGAAGCTTCC TAGGTATGTAGGT TGGACAAAAT CTGC, 3′CAST2 CGTCTAAAAC AGGTTGGATG TATGGATCCT TCGAAGAGCT CGCCTTA, 5′CAST3 AGAAGCTTCC TAGGTGCAGG ACTGGAGAAG TCCTG, 3′CAST3 GTCCTGAAGA GGTCAGGACG TGGATCCTTC GAAGAGCTCG CCTTA, 5′CAST4 ATTCCGCTCG AGAAGCTTCC TAGGGCTATC CAAGTCTTCT GAGAG, 3′CAST4 GAGAGTCTTC TGAACCTATC GGGATCCTTC GAAGAGCTCG CCTTA. Inhibitory domains 1 and 4 (CAST1 and 4, respectively) and CAST1–4 were each cloned into the NdeI and XhoI sites of the pET24d expression vector (Novagen), and inhibitory domains 2 and 3 (CAST2 and 3, respectively) were each cloned into the NcoI and XhoI sites of the pET24a expression vector (Novagen). Both expression vectors provided a C-terminal histidine tag. Using the AvrII and XhoI sites of the clones, the DNA fragment coding for the Ecoil peptide was PCR amplified and inserted between the inhibitory domain and the histidine tag [16].

2.2. Protein expression and purification

Recombinant inactive m-calpain with an N-terminally truncated small subunit (C105S 80 k/21 k) was expressed and purified as previously described [17]. Plasmids containing the CAST1 through 4 + Ecoil and CAST1–4 + Ecoil were transfected into *Escherichia coli* BL21(DE3) (Novagen) by electroporation. LB media (25 ml) was inoculated from a single colony for growth overnight under kanamycin selection before seeding 1 L of LB/kanamycin broth. The broth was grown to an OD_{600} of 0.8–1.0 at 37 °C. Protein expression was induced by addition of 0.4 mM IPTG for 3 h. The cells were collected, resuspended in lysis buffer (25 mM Tris–HCl at pH 7.6, 5 mM EDTA, 5% glycerol, 10 mM β-mercaptoethanol, 100 μM phenylmethylsulfonyl fluoride) and lysed through sonication. The soluble fraction was heated to 85 °C for 15 min. Insoluble matter was removed by centrifugation, and the soluble fraction applied to a 5 ml-Ni^{2+}-chelating agarose resin column (Qiagen). The column was washed with two column volumes of 25 mM Tris–HCl at pH 7.6, 2% glycerol, 500 mM NaCl buffer containing 5 mM imidazole and eluted with the same buffer containing an additional 250 mM imidazole. The CAST-containing fractions, identified using A_{280} and SDS–PAGE analysis, were aliquoted and flash frozen. Each inhibitory domain was purified in a similar manner.

2.3. Expression and purification of CAST1–4

CAST1–4 + Ecoil was produced in the same way as the individual inhibitory domains, except that the heat-treated supernatant was filtered through a 0.45 μm filter and fractionated by Sephacryl S-300 (Pharmacia) size-exclusion chromatography on a 120 ml HiPrep 16/60 column eluted with 25 mM sodium acetate (pH 4.5) containing 0.5 M NaCl. Fractions containing CAST1–4 were identified using SDS–PAGE analysis. Some of the CAST1–4 aggregated and precipitated under these conditions and was recovered by centrifugation at 16000 rpm in a Beckman JA25.5 rotor for 45 min. The pellet was resuspended in denaturing buffer (8 M Urea, 25 mM Tris–HCl pH 7.6, 100 mM NaCl, 2% glycerol, 5 mM imidazole). The resuspended CAST1–4 was applied to 5 ml of Ni^{2+}-chelating resin, washed with denaturing buffer followed by 10 column volumes of non-denaturing buffer (lacking urea), and batch eluted with 250 mM imidazole (pH 7.6) in the same buffer.

2.4. Real-time binding analysis using biosensor technology

The Ca^{2+}-dependent binding of inactive rat m-calpain (C105S 80 k/21 k) to the different CAST constructs was performed using the BIAcore 3000 Surface Plasmon Resonance Spectrometer (GE Healthcare). Kcoil peptide was immobilized onto the surface of a CM5 biosensor chip via a thio-ether bond from the N-terminal cysteine using maleimide coupling [18]. Calpastatin-Ecoil constructs were then injected and bound to the surface of the chip via the Ecoil:Kcoil interaction at a density of about 30 RU [16]. Injections were done at a flow rate of 20 μl/min at a concentration of approximately 10 ng/ml, in the BIAcore running buffer HBS-EP (10 mM HEPES–HCl pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.0005% v/v surfactant P20 (BIAcore Life Sciences)). To monitor the association and dissociation of calpain with calpastatin, the running buffer was modified to include 1 mM DTT and a 1 mM excess of $CaCl_2$ over EDTA, which were at concentrations of 4 mM and 3 mM, respectively. Calpain was diluted from a 3 mg/ml stock in HBS-EP to concentrations 10-fold higher than were tested, and then diluted 10-fold in calcium-containing buffer immediately prior to injection. Injections were performed at a flow rate of 40 μl/min with a 300 s association period and a minimum of 200 s for dissociation. The chips were regenerated by injecting HBS-EP at a flow rate of 40 μl/min until a baseline was reached.

3. Results

3.1. Purification of calpastatin inhibitory domains

During its purification, CAST1–4 precipitated in the fractions from size-exclusion chromatography. This precipitate was recovered by centrifugation and refolded on the Ni^{2+}-agarose column. Although much of the CAST1–4 remained in the supernatant, this fraction was sacrificed because it was contaminated by calpastatin degradation products that were difficult to remove. The individual inhibitory domains were substantially purified by the two-step heat-denaturation and nickel affinity chromatography procedure. Further chromatography was unnecessary because the binding between the Ecoil-tagged CAST and the Kcoil on the chip served as a final highly specific purification step.

3.2. Determination of the binding constants of the calpastatin inhibitory domains for m-calpain

The main objective of this study was to measure the affinity of each of the four inhibitory domains for m-calpain using surface plasmon resonance. This method allowed direct observation of the association (k_{on}) and dissociation (k_{off}) of inactive