Electrostatic interactions of domain III stabilize the inactive conformation of μ -calpain

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The ubiquitous μ - and m-calpains are Ca^{2+} -dependent cysteine proteases. They are activated via rearrangement of the catalytic domain II induced by cooperative binding of Ca^{2+} to several sites of the molecule. Based on the crystallographic structures, a cluster of acidic residues in domain III, the acidic loop, has been proposed to function as part of an electrostatic switch in the activation process. Experimental support for this hypothesis was obtained by site-directed mutagenesis of recombinant human μ -calpain expressed with the baculovirus system in insect cells. Replacing the acidic residues of the loop individually with alanine resulted in an up to 7-fold reduction of the half-maximal Ca^{2+} concentration required for conformational changes (probed with 2-p-toluidinylnaphthalene-6-sulphonate fluorescence) and for en-

zymic activity. Along with structural information, the contribution of individual acidic residues to the Ca^{2+} requirement for activation revealed that interactions of the acidic loop with basic residues in the catalytic subdomain IIb and in the pre-transducer region of domain III stabilize the structure of inactive μ -calpain. Disruption of these electrostatic interactions makes the molecule more flexible and increases its Ca^{2+} sensitivity. It is proposed that the acidic loop and the opposing basic loop of domain III constitute a double-headed electrostatic switch controlling the assembly of the catalytic domain.

Key words: acidic loop, basic loop, Ca^{2+} requirement, electrostatic switch, domain III, μ -calpain.

INTRODUCTION

The superfamily of calpains comprises a number of Ca^{2+} -dependent cysteine proteases and related proteins [1]. μ -Calpain and m-calpain, the 'classical' calpains, have been studied over almost 30 years. These two calpains are expressed in most mammalian tissues, together with their endogenous protein inhibitor, calpastatin. The calpain/calpastatin system seems to participate in important cellular processes like cytoskeleton remodelling, various signal transduction pathways and apoptosis. Although the calpains have been intensely investigated, their physiological roles and mechanisms of regulation are still largely unknown (see [2] for a recent review).

μ -Calpain and m-calpain are heterodimers composed of distinct but homologous large subunits (80 kDa) harbouring the papain-resembling catalytic domain II and a common small subunit (28 kDa) with proposed regulatory functions. Each subunit contains a C-terminal calmodulin-like domain (IV or VI respectively) with 5 EF-hand Ca^{2+} -binding sites. The moderate sequence differences between the large subunits of both isoforms (55–65 % sequence homology) must be responsible for major differences in the Ca^{2+} concentrations required for activity *in vitro* (3–50 μM for μ -calpain and 0.4–0.8 mM for m-calpain [2]), which are far above the free Ca^{2+} concentrations available in living cells (50–300 nM). How the calpains are activated by physiological cellular Ca^{2+} concentrations remains a challenging open question. Autolysis, interactions of the calpains with substrates, activator proteins and/or membrane phospholipids as well as phosphoryl-

ation by protein kinases have been suggested as factors lowering the Ca^{2+} requirement of the calpains to cellular Ca^{2+} concentrations [2].

Crystallographic structures of Ca^{2+} -free m-calpain [3,4], and recently of a ' μ -like' chimaeric μ -m-calpain [5] have explained the inactivity of calpains in the absence of Ca^{2+} by the disruption of the papain-like catalytic domain II into two subdomains IIa and IIb keeping the catalytic Cys far apart from the catalytic histidine residue. Owing to experimental problems resulting from autolysis, aggregation and subunit dissociation [6], attempts to crystallize a complete calpain heterodimer in the presence of Ca^{2+} have so far not been successful. However, crystallography of the isolated protease core (domain II) of μ -calpain [7] and of m-calpain [8] provided the structures of a catalytic domain with weak Ca^{2+} -dependent activity. The conformational changes leading to the fusion of the two subdomains and the formation of a functional active site are effected by a Ca^{2+} switch involving cooperative binding of two Ca^{2+} ions, one on each subdomain. These Ca^{2+} -dependent conformational changes seem to be facilitated in the isolated protease core, because it is freed from structural restrictions imposed by other domains in the context of an intact calpain molecule [9].

Based on the crystallographic structures, two research groups have hypothesized on the forces that keep the subdomains apart in the Ca^{2+} -free enzyme and the mechanisms by which these forces are overcome upon activation with Ca^{2+} [3,4,10]. Owing to its central position, domain III is expected to play a major role in the activation process. The topology of domain III weakly

Abbreviations used: AcNPV, *Autographa californica* nuclear polyhedrosis virus; AMC, 7-amino-4-methylcoumarin; BC-peptide, amino acid residues (one-letter code) ¹⁷⁹GPEVS... to ...PPQEK²⁷⁷ of the calpastatin domain 1 fragment; C115A etc., one-letter amino acid coding denoting Cys-115 mutated to alanine etc.; [Ca^{2+}]_{0.5}, Ca^{2+} concentration for half-maximal effect; CD1, calpastatin domain 1; His₆, hexahistidine (tag); Ni-NTA, Ni²⁺-nitrilotriacetate; Sf, *Spodoptera frugiperda*; TNS, 2-p-toluidinylnaphthalene-6-sulphonate; WT, (recombinant) 'wild-type' μ -calpain.

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Figure 1 Construction of baculoviruses and mutations in domain III

Two *Autographa californica* nuclear polyhedrosis viruses coding for the active (AcNPV-hCAPN1-80K) or for the active-site C115A-mutated [AcNPV-hCAPN1-80K(C115A)] large subunit and one coding for the small subunit (AcNPV-hCAPN4-28K) were prepared for expression of heterodimeric μ -calpain controlled by the polyhedrin (polyHn) promoter. Both forms of the large subunit were mutated within the acidic loop of domain III as shown in the upper part of the scheme.

resembles that of C2-domains that are involved in Ca^{2+} -dependent membrane binding and activation of intracellular signalling proteins like phospholipases and protein kinases [11]. Interestingly, it has been shown that an enzymically active μ -calpain domains I–III construct, overexpressed in a mammalian cell line, translocates to membranes after treatment of the cells with the Ca^{2+} -ionophore, ionomycin, whereas a protease core construct lacking domain III is also active but accumulates in the nucleus [12]. Moreover, Ca^{2+} -binding and Ca^{2+} -dependent binding to phospholipids has been described for an isolated domain III expressed in *Escherichia coli* [13,14].

A striking feature of domain III is its so-called acidic loop (Figure 1), a stretch of consecutive aspartic acid and glutamic acid residues forming salt bridges and/or weaker electrostatic interactions with basic lysine or arginine residues located on domain IIb and on other parts of domain III. It has been proposed that the acidic loop is part of an electrostatic switch mechanism controlling the fusion of subdomains IIa and IIb [4,10]. Disruption of electrostatic interactions would relieve the tension on subdomain IIb and allow the subdomains to fuse. Very recently, experimental evidence has been provided that disruption of some of these electrostatic interactions by site-directed mutagenesis results in an increased calcium sensitivity of calpain [14]. Differences in the electrostatic potentials of the acidic loops and their basic interaction partners could also explain the different Ca^{2+} requirement for the activation of μ -calpain and m-calpain [10], although a different calcium sensitivity seems to be inherent in the isolated protease cores of μ - and m-calpain [9] and the μ -m difference in Ca^{2+} requirement has been attributed to the N-ter-

minus (20%) and to domain IV (65%) in studies with μ -m chimeras [15]. Otherwise, the absence of an acidic loop within the calpain-like domains I–III module of the maize DEK1 protein would explain its activity without calcium [16].

So far, however, it remains unclear whether the electrostatic switch is operated by transduction of the small Ca^{2+} -induced movements originating in the calmodulin-like domain IV and/or is affected by direct binding of Ca^{2+} ions to the acidic loop. In the latter case, incompletely occupied calcium coordination spheres might attract phosphatidyl groups from negatively charged phospholipids and initiate the attachment of the acidic loop to membranes [4]. Conversely, Ca^{2+} -mediated interaction of the acidic loop with membrane phospholipids would enhance Ca^{2+} -binding, and thus lower the Ca^{2+} requirement for activation as it has been suggested in various earlier reports [2].

To address these open questions, we prepared a series of mutants of human μ -calpain in which the acidic residues of the acidic loop were systematically replaced by alanine ('Ala-scan'). Complete heterodimeric μ -calpains containing these mutations were expressed with the baculovirus system in insect cells, both as active enzymes and as inactive C115A (with Cys-115 mutated to alanine) mutants [17,18]. The inactive mutants were used to study the initial Ca^{2+} -induced conformational changes of the intact enzyme in the absence of autolysis, because autolysed calpain is more Ca^{2+} -sensitive and inactivates rapidly [19]. In this paper we report the effects of acidic loop mutations on the Ca^{2+} requirement for conformational changes of the active-site mutated enzyme and for enzymic activity of the active enzyme. In both cases the disruption of single acidic loop interactions leads to a significantly (up to 7-fold) reduced Ca^{2+} requirement. The reduction in Ca^{2+} requirement correlates with the strength of electrostatic interactions predicted from the distances of charged side chains in the structure. Based on these findings, we propose a hypothetical model for the role of the acidic loop and of whole domain III in calpain activation.

EXPERIMENTAL

Materials

ExpandTM High Fidelity DNA polymerase, restriction endonucleases and other DNA modifying enzymes were purchased from Roche (Mannheim, Germany). Oligonucleotides were synthesized by MWG Biotech (Ebersberg, Germany), Roth (Freiburg, Germany) and Metabion (Martinsried, Germany). Protocols, media, and reagents for heterologous protein expression in *Spodoptera frugiperda* (Sf) insect cells using the baculovirus system were from Pharmingen (BD Biosciences, Heidelberg, Germany) and Invitrogen (Groningen, The Netherlands). Native μ -calpain was isolated as described previously [20]. A semi-synthetic gene coding for the 80 kDa subunit of human μ -calpain [12] was a gift from Dr. Shirley Gil-Parrado (University of Munich, Germany). cDNA of the large and the small subunit were generously donated by Professor Koichi Suzuki (University of Tokyo, Tokyo, Japan). 2-p-toluidinylnaphthalene-6-sulphonate (TNS) was purchased from Molecular Probes (Leiden, The Netherlands). The Suc-Leu-Tyr-AMC (7-amino-4-methylcoumarin) substrate was from Bachem (Heidelberg, Germany). Reagent-grade chemicals were obtained from Merck (Darmstadt, Germany) or Sigma (Taufkirchen, Germany) unless otherwise indicated.

Construction of baculovirus transfer vectors

cDNA fragments corresponding to both the 80 kDa and the 28 kDa subunits of human μ -calpain were amplified by PCR and

Limited proteolysis

Proteolytic digestion of the inactive C115A μ -calpain variants (3 μ M) with native μ -calpain from human erythrocytes or chymotrypsin was performed in a volume of 120–150 μ l at room temperature in the presence of either 1 mM CaCl_2 or 1 mM MgCl_2 . Molar ratios of C115A μ -calpain to native μ -calpain and chymotrypsin were 10:1 and 100:1 respectively. Aliquots were removed after specific time intervals for analysis by SDS/PAGE (12.5% gel) followed by Coomassie Blue staining. Reactions were stopped by heating at 90 °C for 5 min after addition of $\frac{1}{4}$ volume of 4 $\times$ SDS sample buffer and EDTA to a final concentration of 2 mM.

N-terminal sequence analysis

The generated C115A μ -calpain fragments were separated by SDS/PAGE, blotted onto a PVDF membrane of 0.1 μ m pore size (Millipore, Bedford, MA, U.S.A.) and stained with Coomassie Blue. N-terminal sequence analysis was performed by Reiner Mentele at the Max Planck Institute for Biochemistry in Martinsried, Germany, using automated Edman degradation on a gas-phase Sequenator 473 (Applied Biosystems, Weiterstadt, Germany).

Ca^{2+} -dependent fluorescence changes

Ca^{2+} -induced intrinsic tryptophan fluorescence changes (excitation at 280 nm) of active-site mutated wild-type (WT) μ -calpain and variants (0.1 μ M) were measured at 22 °C in a buffer containing 50 mM Tris/HCl, pH 7.5, 200 mM NaCl, 0.1 mM EGTA, 5 mM 2-mercaptoethanol, using a Spex FluoroMax fluorescence spectrophotometer (Jobin-Yvon, Edison, NJ, U.S.A.) equipped with a stirrer-adapted thermostatically controlled cuvette holder. Small volumes of a CaCl_2 stock solution were added to the permanently stirred solution every 2 min and fluorescence intensity was recorded after 1.5 min incubation. The increment of the reaction volume was kept < 1% of total volume (3.0 ml). Control measurements were performed with MgCl_2 in place of CaCl_2 . The normalized maximum fluorescence intensity data (emission at 340 nm) were fitted with a modified Hill equation:

$$y = F_0 + F_m \cdot [x^h / (x^h + [\text{Ca}^{2+}]_{0.5}^h)] \quad (1)$$

where y is fraction of maximum fluorescence, F_0 is fluorescence in the absence of Ca^{2+} , F_m is maximum fluorescence change achieved by Ca^{2+} saturation, x is actual free Ca^{2+} concentration (μ M), h is Hill coefficient, and $[\text{Ca}^{2+}]_{0.5}$ is calcium concentration for half-maximal fluorescence changes [26].

Ca^{2+} -induced changes of TNS fluorescence (excitation at 320 nm) were analysed using the same equipment and similar conditions. Measurements were performed in the assay buffer described above both in the absence and in the presence of 200 mM NaCl. A 12-fold molar excess of TNS was mixed with the assay buffer before adding calpain (0.1 μ M) to a final volume of 3.0 ml. Admixing of CaCl_2 or MgCl_2 , data collection (emission at 435 nm) and processing were performed as described above.

Ca^{2+} -dependent substrate hydrolysis

Calpain activity of Sf21 cell lysates obtained as described above was measured in 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol with the fluorogenic substrate Suc-Leu-Tyr-AMC 500 μ M and varying concentrations of CaCl_2 (0–1000 μ M free Ca^{2+}) in a final volume of 500 μ l. The temperature of the assay was kept at 12 °C to slow down autolysis [27]. Release of the fluo-

Figure 2 Purity and activity of heterodimeric μ -calpain variants

(A) SDS/PAGE analysis of inactive C115A mutant (WT C115A) and the inactive acidic loop mutants (as indicated) purified on a calpastatin BC-peptide affinity column. The two bands represent the large (80 kDa) and the small (28 kDa) subunit of the calpain heterodimer. (B) Casein zymogram of soluble supernatants from lysates of cells overexpressing active μ -calpain WT and the active acidic loop mutants (as indicated in A). The calpains were detected by their Ca^{2+} -induced activity bands.

rescent reaction product AMC was monitored in a Kontron SFM-25 fluorimeter (Kontron Instruments, Eching, Germany) (excitation at 380 nm, emission at 460 nm). After 20–40 min, CD1 prepared as described above was added (100 nM) and the AMC release followed for another 15 min. Data were digitally collected using self-programmed software [28]. Calpain activity ($\text{nM} \times \text{min}^{-1}$) was calculated from the initial rate (v_i 0.5–5 min) by subtracting residual proteolytic activity (v_r approx. 10%) which was not inhibited by CD1. Assays were also evaluated after the slope of AMC release had reached a maximum (v_m), subtracting again residual activity as above. Normalized rates and the corresponding free Ca^{2+} concentrations were fitted by non-linear regression analysis with the modified Hill equation:

$$y = A_{\max} \cdot [x^h / (x^h + [\text{Ca}^{2+}]_{0.5}^h)] \quad (2)$$

where $y = (v_i - v_r)/A_{\max}$ and $A_{\max}$ is maximum activity at saturating Ca^{2+} concentration.

Modelling and visualization of structures

A model of WT μ -calpain was obtained from Swiss-Model [29] using the structure of rat μ -like calpain (1qxpB; [5]) as a template. This model and the structure of human m-calpain (1kfu; [4]) were visualized and analysed with Accelrys ViewerLite 5.0.

RESULTS

Structural and functional integrity of the acidic loop mutants

Seven of the eight acidic residues of the acidic loop of domain III were individually replaced by alanine within the large subunit of human μ -calpain (80 kDa) and within an enzymically inactive C115A variant thereof (Figure 1). Together with the complete small subunit (28 kDa), the WT and mutated large subunits were expressed with the baculovirus system in insect cells.

The heterodimeric inactive C115A μ -calpain variants were isolated in > 90% purity by one-step purification on an affinity column with the immobilized BC-peptide fragment of CD1 [23,30] (Figure 2A). Typically, approx. 5 mg of each mutant were

Figure 3 Validation of acidic loop mutants

(A) SDS/PAGE analysis of C115A μ -calpain variants treated with native μ -calpain from human erythrocytes in the presence of Ca^{2+} showing the time course for the WT C115A (left-hand panels labelled 0'–90') and the patterns obtained after 90 min incubation of the acidic loop mutants (right-hand panels labelled D402A etc.). N-terminal sequences of the main cleavage products are indicated on the right-hand side. M, protein size markers. (B) SDS/PAGE analysis of WT C115A and C115A acidic-loop mutants before and after 90 min proteolysis with chymotrypsin, in the presence of 1 mM CaCl_2 (upper panel) or 1 mM MgCl_2 (lower panel). The cartoons on the right-hand side depict schematic structures of Ca^{2+} -activated (upper) and Ca^{2+} -free (lower) calpain. Arrows indicate two different proteolytic fragments with their N-terminal sequences and the corresponding cleavage sites on the large subunit.

obtained from 150 ml suspension cultures containing approx. 1×10^8 infected *Sf21* cells.

The structural and functional integrity of the proteolytically inactive mutants was probed by autolysis and limited proteolysis experiments. Incubation of the inactive calpains with active μ -calpain from human erythrocytes in the presence of 1 mM Ca^{2+} resulted in the typical pattern and time course of autolysis as described for μ -calpain [20]. The pattern after 90 min was virtually the same for all mutants and identical with that of WT calpain (Figure 3A). Although the $[\text{Ca}^{2+}]_{0.5}$ values for TNS fluorescence changes were different for the individual mutants (see below), no significant differences in the degradation rates of the inactive calpains were observed by densitometry (results not shown).

Likewise, limited proteolysis with chymotrypsin resulted in very similar cleavage patterns for all mutants (Figure 3B). The patterns are compatible with a cleavage site before Lys-266 that is exposed in the absence and protected in the presence of Ca^{2+} as reported previously for chymotryptic digestion of μ -calpain [31]. Taken together, the results of limited proteolysis confirm that all enzymically inactive acidic loop mutants (i) were properly folded, and (ii) were able to perform the Ca^{2+} -induced conformational changes that are associated with the activation of calpain. This is congruent with the isolation of the mutants by affinity chromatography on a calpastatin fragment and with their reversible, Ca^{2+} -dependent interaction with immobilized CD1 and kininogen domain 2 as analysed by surface plasmon resonance (results not shown).

The corresponding acidic loop mutants of proteolytically active (not active-site mutated) μ -calpain overexpressed in insect cells were detected in the cell lysates by zymography in the presence of Ca^{2+} (Figure 2B), whereas no proteolytically active bands were found in non-infected control cells as well as in the presence of EDTA (results not shown). Attempts to isolate also the active μ -calpain variants on a calpastatin BC-peptide affinity column (see above) failed because severe autolytic degradation occurred after loading the column in the presence of calcium. Likewise, it was not possible to isolate sufficient quantities of undegraded active μ -calpains from the *Sf21* cell lysates by Ni-NTA affinity chromatography via the C-terminal His₆-tag of the large subunit [32]. Even in the presence of EGTA, the purification yield was low due to partial autolytic degradation and partial loss of the small subunit by dissociation (results not shown). Our observations concur with previous reports on difficulties in obtaining proteolytically active μ -calpain from expression systems [2,32]. Thus we decided to use crude insect cell extracts in the experiments with overexpressed active mutants. This was feasible because the *Sf21* cells displayed virtually no endogenous calpain-like activity (see below).

Ca^{2+} requirement for conformational changes of enzymically inactive mutants

It is well established that autolysis lowers the Ca^{2+} requirement for activity compared with non-autolysed calpain [2]. In order to detect Ca^{2+} -induced conformational changes associated with

Figure 4 Ca^{2+} dependence of Trp intrinsic fluorescence

Semi-logarithmic plot of Ca^{2+} titrations of WT C115A μ -calpain and the D405A mutant. Mean values of normalized fluorescence changes (excitation at 280 nm, emission at 340 nm) from three independent experiments were plotted; the lines represent fits of these data with the Hill equation as described in the Experimental section. The calcium concentrations for $[\text{Ca}^{2+}]_{0.5}$ are $139 \pm 18 \mu\text{M}$ for WT and $81.3 \pm 9.3 \mu\text{M}$ for D405A. Fluorescence spectra of heterodimeric WT C115A μ -calpain at increasing Ca^{2+} concentrations (as indicated) are shown in the inset.

activation of the unautolysed enzyme, we measured conformational parameters of the proteolytically inactive (active-site mutated) variants. As expected from published work [7,9,33], we found a Ca^{2+} -dependent decrease in intrinsic tryptophan fluorescence with WT C115A μ -calpain (Figure 4). The $[\text{Ca}^{2+}]_{0.5}$ of $139 \pm 18 \mu\text{M}$ is within the range of the reported Ca^{2+} requirement for activity of unautolysed μ -calpain [34]. $[\text{Ca}^{2+}]_{0.5}$ values for all acidic loop mutants were reduced (see Figure 4 for an example), but due to the weak fluorescence signals (reflecting the net effect of environmental changes of many tryptophan residues within the heterodimer) the observed differences were not significant for all mutants.

Reliable results were obtained with the conformational probe TNS. A Ca^{2+} -induced rise in TNS fluorescence has been reported for m-calpain [33] and may be explained by the exposure of hydrophobic sites during activation [6]. In 50 mM Tris/HCl we observed a Ca^{2+} -dependent increase in TNS fluorescence for WT C115A μ -calpain and all acidic loop variants (Figure 5) that allowed us to calculate $[\text{Ca}^{2+}]_{0.5}$ values (Table 2). Again, the $[\text{Ca}^{2+}]_{0.5}$ value of the WT enzyme corresponds well to the Ca^{2+} requirement for activation of unautolysed μ -calpain, whereas the $[\text{Ca}^{2+}]_{0.5}$ values of all acidic loop mutants are significantly reduced, reflecting an enhanced Ca^{2+} sensitivity. This effect is most pronounced for D405A (4-fold lower $[\text{Ca}^{2+}]_{0.5}$ than WT) which shows an unusually broad binding curve (Figure 5B). All Hill coefficients are above 1, indicating cooperativity of Ca^{2+} -binding [26]. In the presence of 200 mM NaCl, the effect of mutations on the $[\text{Ca}^{2+}]_{0.5}$ values was clearly reduced (results not shown; see Discussion).

Ca^{2+} requirement for substrate hydrolysis by enzymically active mutants

To circumvent the difficulties in isolation of active μ -calpain (as discussed before), the Ca^{2+} requirement of the overexpressed active calpain variants was determined in crude cell lysates prepared in the presence of EGTA. Calpain activity at increasing concentrations of free Ca^{2+} was measured as the rate of Suc-Leu-Tyr-

Figure 5 Ca^{2+} dependence of TNS fluorescence

(A) TNS fluorescence spectra of WT C115A μ -calpain recorded at excitation at 320 nm with increasing Ca^{2+} concentrations (as indicated). (B) Semi-logarithmic plot of Ca^{2+} titrations of WT C115A μ -calpain and C115A acidic loop variants (as indicated). Mean values of the normalized fluorescence changes (excitation at 320 nm, emission at 435 nm) from three independent experiments were plotted. The lines were obtained by fitting these data with the Hill equation. The calculated $[\text{Ca}^{2+}]_{0.5}$ values are shown in Table 2.

Table 2 Ca^{2+} requirement for half-maximal TNS fluorescence changes of μ -calpain WT C115A and acidic loop mutants

TNS fluorescence changes of μ -calpain WT C115A and acidic loop mutants $[\text{Ca}^{2+}]_{0.5} \pm \text{S.D.}$ and Hill coefficients calculated from the data shown in Figure 5(B).

μ -Calpain type	$[\text{Ca}^{2+}]_{0.5} (\mu\text{M})$	Hill coefficient
WT C115A	130.6 ± 3.8	2.6 ± 0.2
D402A	82.8 ± 2.7	2.1 ± 0.1
E403A	66.7 ± 2.5	1.9 ± 0.1
D405A	31.7 ± 3.0	1.3 ± 0.2
D406A	57.7 ± 2.1	2.0 ± 0.1
D408A	73.5 ± 5.6	1.4 ± 0.1
D412A	93.0 ± 4.1	2.1 ± 0.2
E414A	86.5 ± 3.1	1.8 ± 0.1

AMC hydrolysis inhibited by recombinant CD1, a highly specific inhibitor of μ - and m-calpain [2,35] (Figures 6A and 6B). Lysates of non-infected Sf21 insect cells contained virtually no endogenous calpastatin-sensitive peptidolytic activity (results not shown). We found a distinct Ca^{2+} -dependent increase of calpastatin-sensitive activity in the lysates of all cells overexpressing active WT calpain or the active acidic loop mutants (Figure 6C). Although the substrate Suc-Leu-Tyr-AMC is not specific for calpain, the residual calpastatin-insensitive activity (v_i in

Figure 6 Ca^{2+} dependence of peptidolytic activity

(A, B) Suc-Leu-Tyr-AMC hydrolysis by recombinant active μ -calpain WT (A) and D405A mutant (B) with 40 μM free Ca^{2+} at 12 $^{\circ}\text{C}$. [P], product concentration (filled squares) in nM AMC (left ordinate); v , rate of substrate cleavage (open circles) in nM $\times$ min $^{-1}$ (right ordinate); hatched areas: v_i , initial rate; v_m , maximal rate; v_r , residual rate after addition ($\blacktriangledown$) of 100 nM recombinant CD1. (C) Semilogarithmic plot of normalized initial calpain activity of overexpressed WT and domain III mutants at increasing Ca^{2+} concentrations. Each datapoint is the mean from 2 to 4 independent experiments. The lines represent fits of these data with the Hill equation as described in the Experimental section. The resulting $[\text{Ca}^{2+}]_{0.5}$ values are listed in Table 3.

Figures 6A and 6B) was less than 15% of total activity in all experiments.

Hydrolysis of the fluorogenic substrate was measured at 12 $^{\circ}\text{C}$. At this temperature, activation and subsequent inactivation of calpain due to autolysis is slowed down so that the rate of activation can be followed more accurately [27]. Comparison of Figures 6(A) and 6(B) reveals that at the same Ca^{2+} concentration

(40 μM) the D405A mutant is much faster activated (within a few minutes) than the WT enzyme (within >1 h). The Ca^{2+} dependence of activity was determined from the initial rate after Ca^{2+} addition, as well as from the maximum rate before inactivation becomes visible. The initial rate should mainly reflect the Ca^{2+} requirement for activation of the unautolysed enzyme (corresponding to the conformational changes of inactive variants

Table 3 Ca^{2+} requirement for half-maximal initial activity of μ -calpain WT and acidic loop mutants[Ca^{2+}] $_{0.5} \pm \text{S.D.}$ and Hill coefficients calculated from the data shown in Figure 6(C).

μ -Calpain type	[Ca^{2+}] $_{0.5}$ (μM)	Hill coefficient
WT	104.4 $\pm$ 4.9	2.4 $\pm$ 0.22
D402A	44.9 $\pm$ 3.4	2.5 $\pm$ 0.48
E403A	34.9 $\pm$ 2.0	2.0 $\pm$ 0.21
D405A	15.2 $\pm$ 1.1	1.5 $\pm$ 0.12
D406A	24.0 $\pm$ 1.5	2.0 $\pm$ 0.20
D408A	34.1 $\pm$ 0.9	2.3 $\pm$ 0.12
D412A	46.7 $\pm$ 1.1	2.2 $\pm$ 0.11
E414A	48.1 $\pm$ 2.0	3.2 $\pm$ 0.43

described above), whereas the Ca^{2+} requirement for the maximum rate should be lower due to the presence of autolysed calpain with higher Ca^{2+} sensitivity. The calculated [Ca^{2+}] $_{0.5}$ values for WT calpain, 104.4 $\pm$ 4.9 μM from the initial rate and 49.5 $\pm$ 2.9 μM from the maximal rate, reflect this expected difference. The [Ca^{2+}] $_{0.5}$ values based on the initial rates (Table 3) were up to 2-fold lower when compared with the corresponding values determined from TNS fluorescence of the non-autolysing inactive variants (Table 2). These small but significant differences are probably due to the presence of autolysed calpain molecules with increased Ca^{2+} sensitivity even in the initial phase of the activity assay. However, an influence of salt concentrations (100 mM NaCl in the activity assay versus none in TNS fluorescence) or of unknown positive effectors in the soluble fraction of Sf21 cell lysates cannot be excluded.

Table 3 shows that the [Ca^{2+}] $_{0.5}$ values (calculated from the initial rates) for all acidic loop mutants are significantly lower than for the WT enzyme. Similar to the results obtained with TNS fluorescence (see Table 2), D405A and D406A have the lowest Ca^{2+} requirement for peptidolytic activity (7-fold and 4-fold lower than WT).

DISCUSSION

Disruption of electrostatic interactions of the acidic loop lowers the Ca^{2+} requirement for activation

The acidic loop of domain III, a stretch of consecutive aspartic acid and glutamic acid residues, is a well conserved sequence motif in many calpains [1]. From the crystallographic structures of m-calpain it is anticipated that the negatively charged acidic residues exert electrostatic interactions with positively charged lysine and/or arginine residues (Figure 7). A few of these acidic and basic residues are close enough (< 4 Å; where 1 Å = 0.1 nm) to form direct salt bridges between domain III and domain IIb, but also the overall negative and positive electrostatic potential of all involved residues will certainly contribute to mutual attraction [10,36]. Disruption of these electrostatic interactions seems to be a prerequisite for the movement and rotation of domain IIb leading to the assembly of a functional catalytic site [7]. Conversely, disruption of single interactions should alleviate domain movement and possibly lower the Ca^{2+} requirement for the conformational changes associated with activation. Attempting to find experimental evidence for this hypothesis, and to define the contribution of individual acidic residues to the Ca^{2+} requirement, we replaced these residues one by one with alanine. These single mutations did not affect the overall folding and catalytic function of the calpain variants, but reduced the Ca^{2+} requirement for

conformational changes associated with activation and for peptidolytic activity distinctively (see Tables 2 and 3). The [Ca^{2+}] $_{0.5}$ values for the acidic loop mutated heterodimers (in particular for the D405A mutant) are similar to those reported for the rat μ -calpain protease core [7,9]. This observation suggests that in a sequential activation mechanism disruption of electrostatic interactions at the domain IIb–domain III interface might be a pre-requisite for the crucial cooperative Ca^{2+} -binding in the papain-like domain.

As shown very recently for the salt bridges linking domains IV and VI [19], the electrostatic interactions of the acidic loop were very sensitive to ionic strengths not much above the physiological values. The 200 mM NaCl already used in TNS fluorescence measurements (results not shown) reduced the effects of mutations on the [Ca^{2+}] $_{0.5}$ values compared with the values of Table 2. This kind of fine tuning of electrostatic interactions [37] requires further investigation.

Because a crystallographic structure of human μ -calpain is not available, we used a model based on the recently published structure of a μ -like rat calpain chimera [5] to correlate our experimental results with structure (Figure 7A). When compared with the acidic loop region of m-calpain, the acidic loop of μ -calpain has a somewhat more 'twisted' structure, and the potential electrostatic interactions of the loop with basic residues are partially different. The most obvious difference is that in the μ -calpain loop the role of Asp-400 of m-calpain which interacts with Lys-226 is taken over by a basic residue, Arg-413, interacting with Asp-233 in subdomain IIb. With the exception of the favourable intra-loop contacts Asp-402/Arg-400 (μ -calpain) and Glu-392/Lys-390 (m-calpain), the distances of potentially interacting acidic and basic side chains are markedly greater in μ -calpain than in m-calpain and are out of the range (> 4 Å) required for the formation of single stable salt bridges [36]. This difference correlates with a closer positioning of the catalytic Cys-115 S γ and His-272 N δ 1 (6.3 Å) in the 'apo' conformation of μ -like calpain [5] compared with the corresponding atoms of m-calpain (10.5 Å) [3,4], and might, at least partially, contribute to the lower Ca^{2+} requirement of μ -calpain.

All acidic loop mutants of μ -calpain studied in this work have significantly reduced [Ca^{2+}] $_{0.5}$ values relative to the WT enzyme, suggesting that any mutated residue lowers the negative electrostatic potential of the loop and weakens its interactions with basic residues. The most pronounced reduction of [Ca^{2+}] $_{0.5}$ was seen for D405A (15 % of WT) and D406A (23 % of WT) in the N-terminal portion of the loop (see Table 3). Their side chains interact within < 6 Å with Lys-240 on domain IIb, Arg-364 on domain III, and with Lys-517. This latter residue is located in the C-terminal part of domain III forming the 'root' of the transducer (domain III–IV linker). The side chains of the corresponding acidic loop residues in m-calpain, Asp-395 and Glu-396, are > 6 Å apart from Lys-230 and Lys-354; here the closest interdomain contacts, most probably via salt bridges, are made by Asp-397 with Lys-230 and by Asp-400 with Lys-226. Similar to the corresponding μ -calpain residues, Glu-393, Asp-395 and Glu-396 of m-calpain are involved in contacts with Lys-505 and Lys-506 of the 'pre-transducer' region of domain III.

As mentioned in the Introduction, during preparation of this manuscript Alexa et al. [14] have published data on the Ca^{2+} requirement of acidic loop mutants in rat m-calpain and calpain B of *Drosophila melanogaster*. In their work, simultaneous replacement of Glu-396 and Asp-397 of m-calpain by their respective amides reduced the [Ca^{2+}] $_{0.5}$ value for activity to 3 %, and the corresponding mutations in calpain B to 15 % of the wild-type. The data obtained with rat m-calpain and our data achieved with human μ -calpain concordantly demonstrate the impact of

Figure 7 Electrostatic interactions of the acidic loop

Ribbon plots of the acidic loop of (A) human μ -calpain (modelled using 1qxp as template) and (B) m-calpain (1kfu), showing potential interactions with domain IIb and the root of the domain III-IV linker (transducer). The molecules have been rotated by 180° around the vertical axis relative to the 'reference orientation' [10] in Figure 8. Only side chains with interacting charges separated by < 6 Å are shown, and the minimal distances in Å are presented in the table (bottom panel).

electrostatic interactions of the acidic loop on the Ca^{2+} requirement of calpain activation.

The systematic 'Ala-scan' of all but one acidic residues of the acidic loop in our work reveals that mainly two kinds of electrostatic interactions seem to be responsible for the effect on Ca^{2+} requirement (see Figure 7): (i) interdomain contacts between the acidic loop and domain IIb, and (ii) intradomain contacts between the acidic loop and the 'root' of the transducer (domain III-IV linker). Moreover, the contribution of domain III-domain IIb contacts outside the acidic loop has been first demonstrated by Hosfield et al. [38], who found a 50–60% reduction of $[\text{Ca}^{2+}]_{0.5}$ after mutation of Glu-504 in the pre-linker portion of domain III or its main interaction partner Lys-234 in domain IIb.

It seems probable that simultaneous mutation of two or more residues of the acidic loop would further reduce the Ca^{2+} requirement for activation. The m-calpain double mutant E396Q/D397N of Alexa et al. [14] lowered the $[\text{Ca}^{2+}]_{0.5}$ value 36-fold, compared with the 7-fold and 4-fold reduction by the μ -calpain single mutants E405A and E406A in our work, which are in structurally equivalent positions (see Figure 7). However, neither the data of Alexa et al. [14] nor our own data allow a direct comparison of single mutants and the corresponding double mutants that would lead to conclusions on the additivity of the effects. Moreover, Alexa et al. [14] have clearly shown that almost complete deletion of the loop ($\Delta 395$ –404) of rat m-calpain resulted in an enzyme with increased Ca^{2+} requirement exhibiting rapid non-

autolytic inactivation in the assay. They concluded that the acidic loop is essential for activation as well as for the structural integrity of calpain.

Role of domain III as a double-headed 'electrostatic switch' in calpain activation

Our results provide direct experimental evidence that weakening of electrostatic interactions of the acidic loop of domain III with domain IIb by site-directed mutations increases the rate of calpain activation (see Figures 6A and 6B) and lowers the Ca^{2+} requirement for half-maximal activation (see Figure 6C). We therefore propose that release of electrostatic interactions of the acidic loop plays a critical role in the mechanism of Ca^{2+} -induced calpain activation. As anticipated from the crystallographic structures [3,4], domain III seems to act as an electrostatic switch controlling the relative movement of domains IIa and IIb.

Little is known about how this electrostatic switch is operated during calpain activation. It was believed primarily that the conformational changes leading to calpain activation are exclusively induced by the binding of Ca^{2+} ions to the EF hands of the two calmodulin-like domains IV and VI. Meanwhile, two additional Ca^{2+} -binding sites on subdomains IIa and IIb have been identified that are essential for the assembly of a functional active site. Hence, it has become generally accepted that activation of

calpain is a multi-stage process that involves cooperative binding of Ca^{2+} ions at several sites [7].

Based on its weak topological similarity with C2-domains, it has been suggested that domain III would nucleate up to three Ca^{2+} ions. Ca^{2+} -binding to acidic residues of the acidic loop, enhanced by coordination with phospholipids, would weaken their electrostatic interactions, thereby sensitizing the electrostatic switch [10]. Indeed, it has been shown that isolated domains III of rat μ - and m-calpain and of *Drosophila* calpain B expressed in *E. coli* were able to bind calcium ions and that Ca^{2+} -binding was enhanced by phospholipids [13]. Although recombinant domains III are unstable, Alexa et al. [14] demonstrated that domain III of *Drosophila* calpain B binds 2–3 Ca^{2+} ions and that deletion of the acidic loop abolishes Ca^{2+} -binding. The rather high K_d of 3.4 mM for Ca^{2+} -binding to domain III was lowered 15-fold in the presence of phospholipid. The relevance of these intriguing findings for the mechanism of activation of full-size calpains *in vitro* and in living cells remains to be established.

Whereas modulation of the electrostatic switch by direct Ca^{2+} -binding to the acidic loop seems reasonable, the switch is probably geared by conformational changes transmitted from the Ca^{2+} -binding EF hands of domains IV and VI. Alexa et al. [14] provided experimental evidence for the importance of the so-called transducer (domain III–IV linker) which is expected to communicate the small but significant Ca^{2+} -induced conformational changes within domain IV [39] to domain III and may even amplify these small changes via an ‘entropic spring’ mechanism. Moreover, our data suggest that the electrostatic interactions of three acidic residues of the loop (Glu-403, Asp-405, Asp-406) with Lys-517 of the transducer ‘root’ strongly contribute to the Ca^{2+} requirement for activation (see Table 3 and Figure 7A). These interactions stabilize the loop in its ‘resting’ position but may be easily disrupted on small movements of the transducer. The concerted weakening of both loop–domain IIb and loop–transducer interactions could explain the outstanding effects of the D405A and D406A mutations in our work as well as of a E396Q-D397N mutant of rat m-calpain on $[\text{Ca}^{2+}]_{0.5}$ [14].

Opposite to the acidic loop, domain III of calpains has a complementary sequence motif, the basic loop, containing several consecutive lysine and/or arginine residues (Figure 8). Polar contacts of this novel hairpin motif with the $\alpha_5\text{II}$ and $\alpha_6\text{II}$ helices in subdomain IIa were early recognized in the crystallographic work to provide rigidity to subdomain IIa with respect to subdomain IIb [4]. The importance of these interactions is underlined by the observation that mutations of three of these basic residues to tryptophan in the corresponding loop of muscle-specific calpain 3 (p94) lead to enzyme dysfunction in limb-girdle muscular dystrophy type 2A patients [40,41]. Yet, not much attention has been paid to the fact that these basic residues closely interact with acidic residues involved in Ca^{2+} -binding by the first two EF hands of domain VI of the small subunit in the heterodimeric μ - and m-calpains [8]. Disruption of these electrostatic contacts by Ca^{2+} -binding or, more than ever, by dissociation of the small subunit will ‘unlock’ domain III and increase its overall mobility relative to the other domains. Interestingly, partial and complete deletion of the involved EF hand motifs resulted in decreased specific activity and heterodimer instability in rat m-calpain [42]. The effect of these mutations was enhanced by high ionic strengths, a factor known to weaken electrostatic interactions [36]. An increase in the Ca^{2+} requirement for activity of m-calpain was measured when the Ca^{2+} -binding capacity of the mentioned EF-hands was ablated by site-directed mutagenesis [39]. In addition, this region has been shown to be the docking site of calpastatin C-peptide [43], a further indication of the importance of domain VI–domain III interactions.

Figure 8 Domain III as double-headed electrostatic switch

Ribbon plot of human m-calpain (1kfu) showing the electrostatic interactions of the acidic loop (Glu-392–Gly-404) and the basic loop (Lys-414–Glu-424) of domain III with other parts of the molecule (within the solid circles). Interacting acidic (grey) and basic (black) side-chains separated $< 6 \text{ \AA}$ are presented as sticks. The broken circle encloses electrostatic interactions between domain IV and the transducer (domain III–IV-linker).

In summary, multiple electrostatic interactions of domain III with the catalytic domain II stabilize the inactive conformation of μ -calpain. During activation of calpain by Ca^{2+} ions, domain III seems to function as a double-headed electrostatic switch controlling the assembly of the active site by gradual disruption or rearrangement of these interactions (Figure 8). This electrostatic switch could be effectively actuated by simultaneous Ca^{2+} -binding to domain IV (via the transducer) and to domain VI (via the basic loop). Although none of the point mutations described in our work lowered the Ca^{2+} requirement of μ -calpain even close to physiological cellular Ca^{2+} concentrations, one may expect that concerted weakening of several interactions within the electrostatic switch would effectively reduce the Ca^{2+} requirement for activation, eventually down to the cytosolic Ca^{2+} levels. It is not known whether and how these changes are achieved in living cells. The electrostatic switch could be ‘sensitized’ via charge neutralization by the binding of Ca^{2+} ions coordinated with membrane phospholipids (as suggested in [14] and discussed above) or by direct electrostatic interactions with protein substrates and/or activator proteins (see [2] for review). Additionally, local changes in charge density effected by phosphorylation may regulate the activation of calpains in the cell [2,44].

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