Origins of the difference in Ca^{2+} requirement for activation of μ- and m-calpain

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The μ- and m-calpains are closely related Ca^{2+}-dependent cysteine proteases having different *in vitro* Ca^{2+} requirements (K_d), of approx. 25 and 325 μM respectively. The two isoforms are heterodimers of slightly different large (80 kDa) subunits and an identical small (28 kDa) subunit, so that the difference in K_d values must reside in the large subunits. As assayed here, these K_d values relate to the Ca^{2+} required for the first phase of calpain activation and do not reflect the lower Ca^{2+} then required by fully activated calpain. On the basis of sequence comparison and the X-ray structure of m-calpain, many m-type residues in the C-terminal EF-hand-containing domain IV were converted into the corresponding μ-type residues, but these mutations did not produce the expected decrease in K_d. In a series of hybrid (μ/m) large-subunit calpains, the K_d values decreased progressively towards that of μ-calpain as the proportion of μ-type sequence increased from 0 to 90 %. K_d values cannot therefore be ascribed to one or a few specific intramolecular interactions, but reflect the global response of the whole molecule to Ca^{2+} binding. Nonetheless, 25 % of the difference in K_d values between μ- and m-calpain can be ascribed to the N-terminal peptide of the large subunit, whereas the C-terminal EF-hand-containing domain IV accounts for 65 % of the difference.

Key words: EF-hand, hybrid calpain, site-directed mutagenesis.

INTRODUCTION

The two calpains, μ- and m-calpain, are heterodimeric Ca^{2+}-dependent cysteine proteases found in moderate abundance in most mammalian tissues [1–4]. The enzymes differ in their *in vitro* Ca^{2+} requirements, μ-calpain having a K_d value variously reported as 10–50 μM and m-calpain having a K_d value of 300–350 μM. The two enzymes have slightly different large (80 kDa) subunits of approx. 700 residues with an amino acid sequence identity of approx. 62 % and an identical small (28 kDa) subunit of 270 residues. The difference in K_d must therefore lie in the large subunits. The reasons for this difference are not yet known, and we have made a variety of mutations in m-calpain in search of an explanation. To assist in understanding these mutations, a comparison of the primary sequences of the large subunits of μ- and m-calpain is shown in Figure 1, and a domain diagram of calpain derived from the X-ray structure [5,6] is shown in Figure 2. The large subunit consists of a short N-terminal peptide and four domains. Domains I and II comprise the protease core region with the active-site residues. Domain III is a C2-like domain whose function is not clear, and domain IV contains five EF-hand motifs. The small subunit contains two domains, namely a glycine-rich domain V and domain VI, which also contains five EF-hand motifs and is very similar to domain IV. In our recombinant calpains, domain V is normally omitted, since this omission increases expression levels and does not affect K_d values.

The process of calpain activation involves several steps. Binding of Ca^{2+}, at least partly at EF-hands in domains IV and VI, induces a conformational change, leading to modification or release of contacts between the N-terminus of the large subunit and domain VI of the small subunit and also between domain IV of the large subunit and domain VI of the small subunit. Autolysis of the large-subunit N-terminal peptide and of domain V, if it is present, also occurs at about this time and makes the activation process irreversible. Binding of Ca^{2+} at two sites in domains I and II causes conformational changes in these domains, which bring the active-site residues into fully active conformation [7]. The precise sequence of these events has not been established, but it is well known that the initial phase of activation requires a relatively high Ca^{2+} concentration and that the enzymes, once fully activated, continue to require Ca^{2+} for activity, although at a lower level [3]. The constant K_d measured in this work and in previous work on calpain [3,8,9] is defined as the concentration of Ca^{2+} required for 50 % of maximum activity with casein as the substrate, and K_d refers primarily to the initial phase of calpain activation. Our present view is that the lower Ca^{2+} requirement observed after activation is more closely related to the binding sites in domains I and II. *In vivo*, where the available Ca^{2+} levels are apparently insufficient, the activity of calpain is variously explained either by the binding of calpain to phospholipids or activating proteins or by the occurrence of localized high concentrations of Ca^{2+}. These latter questions, although important for biological function, are not directly relevant to the work described here on purified recombinant calpains *in vitro*.

The Ca^{2+}-binding sites detected in domains I and II are essential for maintaining the activity of calpain, but it appears that the primary factor initiating calpain activation is Ca^{2+} binding to EF-hands in domains IV and VI, although the evidence for this idea is indirect. The structure of m-calpain in the absence of Ca^{2+} has been described previously [5,6], but a crystal structure of calpain with bound Ca^{2+} has not been obtained because of technical problems related to dissociation and aggregation of calpain in the presence of Ca^{2+} [10,11]. X-ray crystallography of the isolated domain VI (small subunit) homodimer showed that there was only a very small conformational difference between the Ca^{2+}-bound and Ca^{2+}-free states [12,13]. From this it is assumed that the Ca^{2+}-induced changes in domains IV and VI of the intact enzyme are small, although they are clearly sufficient to initiate calpain activation. Since direct observation is not feasible, the role of Ca^{2+} binding at EF-hands in calpain

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                                20                  40      BamHI
MA.........GIAMKLAKDREAAEGLGSHERAIKYLNQDYETLRNECLEAGALFQDPS
--EELITPVYCT-VSAQVQ-Q-DKEL---R--N-----G----N--AR--QN-V----DA
                  20                  40                  60

        60                  80                  100
FPALPSSLGFKELGPYSSKTRGIEWKRPTEICADPQFIIGGATRTDICQGALGDCWLLAA
--PVSH---------N----Y--K------LLSN----VD--------------------
                  80                  100                 120

       120                 140                 160
IASLTLNEEILARVVPLDQSFQENYAGIFHFQFWQYGEWVEVVVDDRLPTKDGELLFVHS
--------T--H----YG-----G--------L--F----D-----L------K-V----
                  140                 160                 180

       180                 200                 220
AEGSEFWSALLEKAYAKINGCYEALSGGATTEGFEDFTGGIAEWYELRKPPPNLFKIIQK
-Q-N-------------V--S-------C-S-A-------VT---D-Q-A-SD-YQ--L-
                  200                 220                 240

       240                 260                 280
ALEKGSLLGCSIDITSAADSEAVTYQKLVKGHAYSVTGAEEVESSGSLQKLIRIRNFWGQ
---R---------N-SDIR-L--I-FKN--R-------D-KQ-TYQ-QRVN---M-----E
                  260                 280                 300

       300                 320                 340
VEWTGKWNDNCPSWNTVDPEVRANLTERQEDGEFWMSFSDFLRHYSRLEICNLTPDTLTC
---K-P-S--SYE--K---YE-EQ-RVKM---------R--I-EFTK---------A-KS
                  320                 340                 360

       360                 380                 400
DSYKKWKLTKMDGNWRRGSTAGGCRNYPNTFWMNPQYLIKLEEEDE.DDEDG.ERGCTFL
RTLRN-NT-FYE-T--------------A---V---FK-R---V-DA--Y-SR-S--S--
                  380                 400                 420

         420                 440                 460
VGLIQKHRRRQRKMGEDMHTIGFGIYEVPEELTGQTNIHLSKNFFLTTRARERSDTFINL
LA-M------E-RF-R--E----AV-Q--R--A--P.V--KRD---ANAS-AQ-EH----
                  440                 460                 479

         480      SalI       500                 520
REVLNRFKLPPGEYVLVPSTFEPHKNGDFCIRVFSEKKADYQTVDDEIEANI.EEIEANE
---S--IR------IV-------N-E---LL-F------GT-EL--Q-Q--LPD-KVLS-
                  500                 520                 539

          540   EF hand 1      560       FspI       580
EDIGDGFRRLFTQLAG*EDAEISAFELQTILRRVLAKREDIKSDGFSIETCKIMVDMLDE
-E-D-N-KT--SK---*D-M---VK------N-IIS-HK-LRTN---L-S-RS--NLM-R
               DGDG 560                 580                 598

 EF hand 2          610      EF hand 3  630      DraI
DGSGKLGLKEFYILWTKIQKYQKIYREIDVDRSGTMNSYEMRKALEEAGFKLPCQLHQVI
--N-----V--N---NR-RN-LT-F-KF-L-K--S-SA----M---A-----NKK--EL-
                  620                 640                 658

    EF hand 4        670          EF hand 5        700
VARFADDELIIDFDNFVRCLVRLEILFKIFKQLDPENTGTIQLDLISWLSFSVLGKLAAA
IT-YSEPD-AV------C------TM-RF--I--TDLD-VVTF--FK--QLTMFA-----
                  680                 700         712

LEHHHHHH
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Figure 1 Comparison of wild-type amino acid sequences of the large subunits of rat μ- and m-calpain.

The upper line represents m-calpain, whereas the lower line represents μ-calpain. The active-site residues Cys[105]/*Cys*[115], His[262]/*His*[272], Asn[286]/*Ans*[296] have been enclosed in boxes. Residue numbers for m-calpain are indicated above the sequence in roman font and those for μ-calpain in italics. Identical residues are indicated by a dash. Gaps, marked as dots, have been introduced in m-calpain following residues 2, 396, 401 and 520 and in μ-calpain following residue *456*, to optimize the alignment. The Ca^{2+}-co-ordinating loop sequences of EF-hands 1–3 have been underlined. A gap marked as * has been introduced in both proteins following residues 543/*555* in EF-hand 1 to indicate the apparent deletion in this EF-loop sequence as compared with the canonical EF-loop sequence. The separate mutation of EF-hand 1 to replace Ala-Gly-Glu with Asp-Gly-Asp-Gly (to approximate the canonical sequence) is indicated below the section of the sequence (see also Table 3). The positions in the cDNA sequences of four restriction-enzyme sites that were used to construct large-subunit hybrids are indicated above the amino acid sequence.

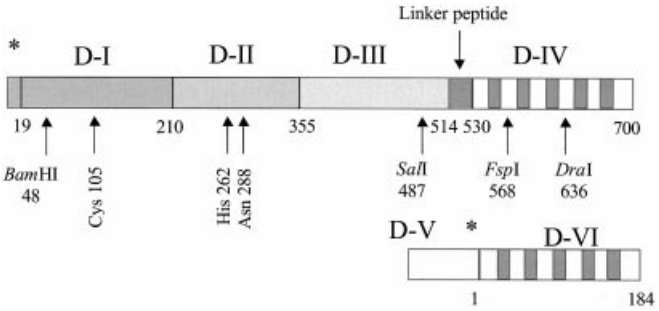

Figure 2 Domains of m-calpain

The approximate positions of the domain boundaries are indicated. Five EF-hands are shown, not to scale, in domain IV (and in domain VI) to indicate their positions relative to the *Fsp*I and *Dra*I sites. Sites of autolysis of the natural enzymes *in vivo* are indicated by * in the N-terminal region of the large subunit and in domain V of the small subunit. Domain V was not present in the recombinant calpains described here. It should be also noted that other workers have defined the N-terminal peptide of the large subunit as domain I and the protease core region (here domains I and II) as domains IIa and IIb [6]. Adapted with permission from Hosfield et al., EMBO J., 1999 [5] © Oxford University Press.

activation was therefore studied by means of mutation [8,9]. Such studies showed that, of the five apparent EF-hand motifs in each subunit, EF-hand 3 appears to have the highest affinity for Ca^{2+} and EF-hands 4 and 5 probably do not bind to Ca^{2+}. Abolition of Ca^{2+} binding at EF-hand 3 simultaneously in both subunits increased K_d value for wild-type m-calpain from the control value of 325 μM to 1.7 mM. Further abolition of Ca^{2+} binding at EF-hands 1–3 simultaneously in both subunits increased the K_d value to 7.4 mM. Since the Ca^{2+} requirement for activation was greatly increased in calpains with defective EF-hands, whereas the Ca^{2+}-binding sites in domains I and II were unchanged, it is evident that Ca^{2+} binding in domains IV and VI is essential for calpain activation.

These results led us to look within domain IV of μ- and m-calpain for an explanation of the difference in K_d. The amino acid sequence identity between domain IV in μ-calpain and domain IV in m-calpain is only 51 %, but the actual Ca^{2+}-co-ordinating residues in EF-hands 1–3 are almost 100 % conserved, hence the difference in K_d value must be ascribed to more subtle factors. It was recognized also that the Ca^{2+} affinity of any EF-hand is a property of the whole helix–loop–helix motif and of its interactions with the rest of the molecule [14], but we hoped nonetheless to identify some crucial intramolecular contacts. Therefore on the basis of sequence comparison and inspection of the m-calpain X-ray structure (since the X-ray structure of μ-calpain is not yet available for comparison), we mutated a variety of domain IV m-type residues to the corresponding μ-type residues. We also constructed a set of hybrid calpain large subunits with different proportions and distribution of μ- and m-type sequences. The results showed that the whole large subunit is involved in determining the K_d value in calpain, but that domain IV makes the largest contribution to this value.

EXPERIMENTAL

Bacterial expression and purification of calpain

The pET-24 plasmid, encoding the rat m-calpain large subunit with a C-terminal His tag, and the pACpET plasmid, encoding the 21 kDa form (domain VI) of the rat calpain small subunit, have been described [15]. The corresponding pET-24 plasmid for the rat μ-calpain large subunit was prepared with the kind assistance of Dr H. Sorimachi [16]. The calpains were obtained by co-expression from large- and small-subunit plasmids in *Escherichia coli* strain BL21(DE3). The cells were grown at 30 °C to an D_{600} of approx. 1.0, calpain synthesis was induced with isopropyl β-D-thiogalactoside and growth was continued for 16 h at 25–27 °C.

The calpains were routinely purified from the sonicated lysate obtained from *E. coli* grown in 4–8 litres of culture, by

chromatography successively on columns of DEAE–Sepharose, Ni^{2+}-nitrilotriacetate–agarose and Q-Sepharose [15,17]. For well-expressed and active calpains, a shorter and simpler purification procedure from *E. coli* grown in a 500 ml culture was found to be satisfactory. This involved a small Q-Sepharose column with a salt gradient followed by batch absorption and elution from Ni^{2+}-nitrilotriacetate–agarose, and it provided enough enzyme of sufficient purity for Ca^{2+} titration.

One unit of calpain activity is the amount of enzyme causing a Ca^{2+}-dependent increase in A_{280} of 0.1 in 30 min at 25 °C, under the assay conditions described previously [8,9]. This unit represents 0.5–1 μg of the various purified recombinant calpains.

Site-directed mutagenesis

Three distinct sets or categories of mutations were introduced into μ- and m-calpain cDNA using single-strand DNA by the method of Kunkel [18], and all constructs were checked by restriction digestion and sequencing. In the first set, since there are only a few differences between μ- and m-calpain in the Ca^{2+}-co-ordinating loops of EF-hands 1–3, these m-type residues were mutated (as shown bold in Figure 1), one EF-hand at a time, to the specific characteristics of μ-calpain. Secondly, some small m-to-μ peptide substitutions were made in domain IV, at positions involving intramolecular salt links or hydrophobic interactions, which appeared to differ significantly between μ- and m-calpain. These were: (a) replacement of five m-calpain residues 517–521 (Glu-Ala-Asn-Ile-Glu) by the corresponding six μ-calpain residues *528–533* (*Gln-Ala-Asn-Leu-Pro-Asp*); (b) replacement of three m-calpain residues 639–641 (Pro-Cys-Gln) by the corresponding three μ-calpain residues *651–653* (*Asn-Lys-Lys*); (c) replacement of two m-calpain residues 578–579 (Lys-Ile) by the corresponding μ-calpain residues *590–591* (*Arg-Ser*).

Finally, in order to permit the exchange of large polypeptide segments, restriction sites were introduced (in most cases to match an existing site in the other isoform) as follows: (a) μ-calpain, *Bam*HI (*Gln*^{57}, *Asp*^{58}, *Asp*^{59}→*Pro*); *Dra*I (*Phe*^{648}, *Lys*^{649}, silent); (b) m-calpain, *Sal*I (Pro^{486}, Ser^{487}, Thr^{488}, silent); *Fsp*I (Ile^{567} → Leu, Lys^{568} → Arg, Ser^{569} → Thr); *Dra*I (Phe^{636}, Lys^{637}, silent). These restriction sites are also shown in Figure 1. For the non-silent mutations listed, it was shown that the mutations alone did not affect the expression and K_d values of the resultant calpains (results not shown). For μ-calpain, the amino acid residue names and numbers are given in italics throughout this paper.

Generation of hybrid calpain large subunits and their nomenclature

Using the unique *Bam*HI, *Sal*I, *Fsp*I and *Dra*I restriction sites in μ- and m-calpain large subunit cDNA, hybrid subunits were generated by standard cloning procedures and checked by sequencing. These large subunits are described for brevity in terms of 'm' and 'μ' and the restriction sites: e.g. the construct m-Bam-μ-Fsp-m contains m-calpain residues 2–49, μ-calpain residues *60–581* and m-calpain residues 570–714. The exact sequences and their abbreviations are listed in Table 1. For enzymic analysis, the calpains were expressed as 80 + 21 kDa heterodimers, but again for brevity the enzymes are named only in terms of their large subunits.

Heat-stability

Purified calpains (150 μg/ml, approx. 300 units/ml) were dialysed against 50 mM Tris/HCl (pH 7.6)/0.2 M NaCl/2 mM EDTA/10 mM 2-mercaptoethanol. Samples were incubated at 45 °C and the aliquots were returned to ice at intermediate times up to 40 min. At the end of this treatment, the calpain activity remaining in all the samples was assayed in duplicate using the standard casein assay. A sample of wild-type m-calpain was analysed in parallel as a control.

Table 1 Abbreviations for hybrid calpains and their amino acid sequences

Abbreviation	Amino acid sequence*
Wild-type m-80	m-2-714
m-Bam-μ-Sal-m	m-2-49, μ-*60-498*, m-488-714
m-Bam-μ-Fsp-m	m-2-49, μ-*60-581*, m-570-714
m-Bam-μ-Dra-m	m-2-49, μ-*60-649*, m-638-714
m-Bam-μ	m-2-49, μ-*60-726*
μ-Bam-m	μ-*2-58*, m-49-714
μ-Sal-m	μ-*2-498*, m-488-714
μ-Fsp-m	μ-*2-581*, m-570-714
μ-Dra-m	μ-*2-649*, m-638-714
Wild-type μ-80	μ-*2-726*
m-Sal-μ-Fsp-m	m-2-487, μ-*499-581*, m-570-714
m-Sal-μ-Dra-m	m-2-487, μ-*499-649*, m-638-714
m-Sal-μ	m-2-487, μ-*499-726*
m-Fsp-μ	m-2-566, μ-*579-726*
m-Dra-μ	m-2-637, μ-*650-726*

* Each construct has a 14-residue C-terminal extension, which includes the His_6 tag. μ-Calpain residue numbers are shown in italics.

Casein zymography

Casein zymography was performed as described previously [19,20] and it provided a rapid check that the various mutated and hybrid calpains were active, before proceeding to large-scale expression.

Titration of the Ca^{2+} requirement of mutant calpains for casein hydrolysis

The difficulties involved in obtaining reproducible Ca^{2+} titration curves for calpain, and the statistical considerations, have been discussed previously [8,9]. The titrations were performed under carefully standardized conditions, including at least one control titration, usually of wild-type m-calpain, in each experiment. The S.D. of the K_d derived from a single titration curve was less than 5%, but the wild-type K_d value varied slightly (±10%) from day to day over several months. The values have been normalized to a wild-type K_d value of 325 μM. The K_d values of the wild-type μ-calpain and m-Bam-μ were measured only once. The K_d values for all other enzymes were measured at least twice on separate occasions and on different preparations and the results lie within 10% of each other; the values given are the means for two observations.

RESULTS

Expression and specific activities

Except where indicated, all the mutant calpains described in the present study were well expressed, readily purified and had specific activities within the range of 1–2 units/μg of protein (compared with the wild-type m-calpain value of 1.8 units/μg of

Table 2 Heat-stability of some hybrid calpains

The calpains were dialysed overnight and adjusted to equal protein concentration (150 μg/ml, approx. 300 units/ml). They were incubated at 45 °C in 50 mM Tris/HCl (pH 7.6), 2 mM EDTA, 0.2 M NaCl and 10 mM 2-mercaptoethanol for 40 min, returning aliquots to ice at intermediate times. The remaining caseinolytic activity was assayed. In all cases, a wild-type m-calpain sample was assayed in parallel and the values have been adjusted to 40% of wild-type m-calpain activity remaining after 40 min. The time course of loss of activity was close to linear, so that only the final values at 40 min are given.

Hybrid calpain	Activity remaining (%)
Wild-type m-calpain	40
m-Bam-μ-Sal-m	40
m-Bam-μ-Dra-m	95
m-Sal-μ-Fsp-m	82
m-Sal-μ-Dra-m	70
μ-Sal-m	65
μ-Dra-m	85

Table 3 Ca^{2+} requirement (K_d) of m-calpains having m- into μ-type conversions of the large subunit EF-loop residues

Abbreviated name of construct	Mutated residues	K_d (μM)*
Wild-type m-80-k		325
m-80-k-EF1 m-to-μ	E544D, A546M, A550V, F551K	398
m-80-k-EF1 m-to-consensus	A542D, G543, Δ543aD, E544G†	278
m-80-k-EF2 m-to-μ	E586R, S589N, K595V	363
m-80-k-(EF2 + 3) m-to-μ	E586R, S589N, K595V, V616L, R618K	438
m-80-k-EF3 m-to-μ	T621S, N623S, S624A	473

* The K_d values for each construct were measured separately with a wild-type m-calpain as control and have been normalized to a wild-type value of 325 μM.

† The Ca^{2+}-co-ordinating loop of EF-hand 1 appears to consist of only 11 residues in the place of the normal 12, and the apparent omission is shown as Δ [12] (see also Figure 1). In this construct (converting AGE into DGDG), an additional residue was introduced (a), while replacing the wild-type sequence with a sequence closer to the consensus for EF-hands.

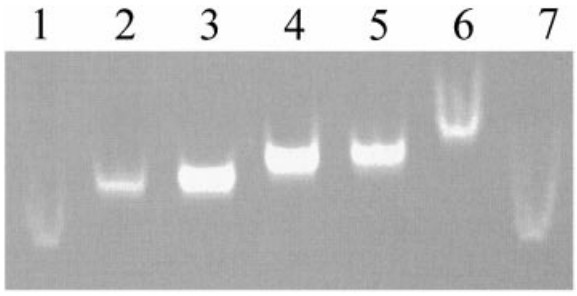

Figure 3 Casein zymogram of some hybrid calpains

The enzymes were expressed as stable heterodimers of 80 + 21 kDa subunits. The lanes were loaded with the soluble supernatant fraction from the sonicated *E. coli* lysates and contained the following: 1 and 7, wild-type m-80; 2, m-Bam-μ-Sal-m; 3, m-Bam-μ-Fsp-m; 4, m-Bam-μ-Dra-m; 5, m-Bam-μ; 6, wild-type μ-80. The proteins migrated vertically towards the bottom.

protein). Hybrid calpains with the C-terminal μ-calpain sequence either were inactive (m-Sal-μ, m-Fsp-μ and m-Dra-μ) as judged by casein zymography, or were too poorly expressed for extensive analysis (wild-type μ-80-k, m-Bam-μ). It appears that the C-terminus of μ-calpain (with or without a His tag) is unfavourable for *E. coli* expression (and apparently also in Sf9 cells [21]) for reasons that remain unclear. In contrast, calpains with at least 65-m-type C-terminal residues (downstream of the *Dra*I site) were well expressed and fully active. Casein zymography also provided some supporting evidence for the identity of the hybrid calpains, since the relative mobility of hybrid calpains on these gels was proportional to the predicted total charge of the large subunit (Figure 3).

Heat-stability

The loss of activity as a function of incubation time at 45 °C in the presence of 0.2 M NaCl and 2 mM EDTA was measured for some hybrid calpains in parallel with a wild-type m-calpain control (Table 2). Their heat stabilities were in general greater than that of wild-type m-calpain and correlated approximately with the fraction of μ-calpain sequence. It has been reported previously that μ-calpain was more heat-stable than m-calpain [22]. The heat-stabilities of the various m-calpains containing point mutations were not significantly different from that of wild-type m-calpain.

Modifications of EF-hands

The Ca^{2+}-co-ordinating loop of each of the EF hands 1–3 was separately converted from the m-type into the μ-type, by changing the residues indicated in bold in Figure 1. The K_d values for these mutant EF-hand calpains are shown in Table 3. The m-into-μ loop conversion in EF-hand 1, and separately in EF-hand 2, in each case yielded an active enzyme, but the enzymes showed modest increases in K_d rather than the anticipated decreases in K_d towards more μ-calpain-like values. The calpain with mutations of all five indicated residues in the loop in EF-hand 3 was inactive, but two partially modified forms of EF-hand 3 were fully active and both of these enzymes also had elevated K_d values. Finally, since the loop in EF-hand 1 is slightly unconventional [12,13], the naturally occurring tripeptide Ala-Gly-Glu (residues 542–544) was replaced with a tetrapeptide Asp-Gly-Asp-Gly, which conforms more closely with the canonical EF-hand loop sequence (Figure 1). This mutation indeed caused a small reduction in the K_d value (Table 3), consistent with an improved EF-hand.

Other localized mutations

Three further mutations were made at positions involving apparently important intramolecular contacts, which differed between μ- and m-calpain. Mutation of m-calpain residues 517–521, Glu-Ala-Asn-Ile-Glu, to *Gln-Ala-Asn-Leu-Pro-Asp*, and of m-calpain residues 639–641, Pro-Cys-Gln, to *Asn-Lys-Lys*, both involving changes in salt links, had no significant effect on the Ca^{2+} requirement of the resultant calpains. The observed K_d values were 335 μM for mutation of residues 517–521 and 350 μM for mutation of residues 639–641. Mutation of m-calpain residues 578 and 579, Lys-Ile, to Arg-Ser, involving a change in a hydrophobic contact, caused an increase in K_d from 325 to 412 μM.

Hybrid calpains

Since the point mutations had caused little change in K_d values, hybrid calpains were constructed in order to localize the K_d-determining regions of the large subunit. A diagram of these constructs is shown in Figure 4(a) together with their observed K_d values. A plot of the K_d values against the m/μ content of the large subunits is shown in Figure 4(b). Some representative Ca^{2+} titration curves are shown in Figure 5.

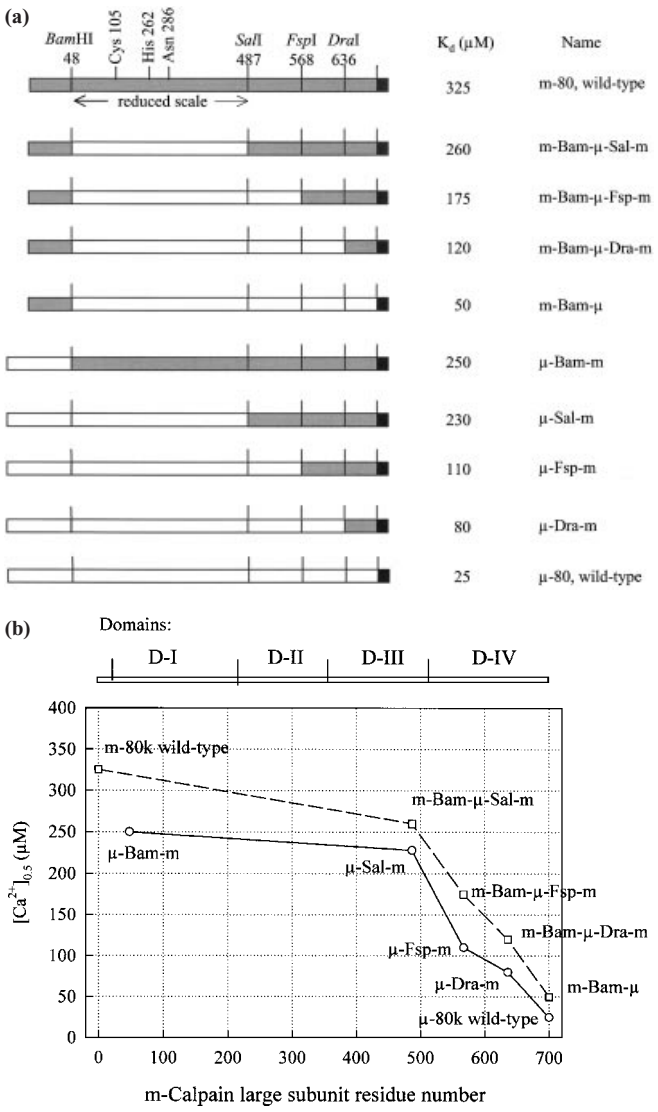

Figure 4 Structures and K_d values of wild-type and hybrid calpains

(**a**) Diagram of the large subunit constructs. Most of the domains I, II and III between the *Bam*HI and *Sal*I sites had relatively little effect on K_d values, and this section has been reduced in scale here. The grey bars indicate m-calpain sequence, the open bars represent μ-calpain sequence and the black bars indicate the C-terminal His-tag region. (**b**) Relative contribution of the domains to K_d values. Plot of K_d against the position in the large subunit of the μ/m fusions. The large-subunit domain diagram is shown again at the top of the Figure.

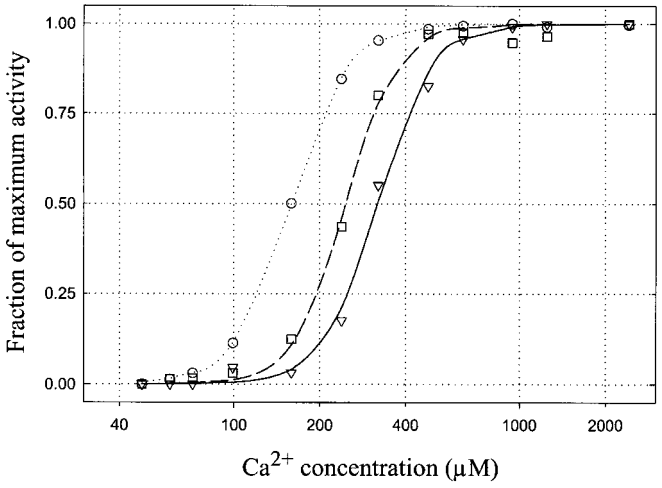

Figure 5 Representative Ca^{2+} titration curves

The three calpains shown were freshly purified, adjusted to a concentration of approx. 300 units/ml and dialysed overnight at 4 °C against 50 mM Tris/HCl (pH 7.5), 0.2 M NaCl, 0.2 mM EDTA, 10 mM 2-mercaptoethanol and, 0.01% sodium azide. Duplicate samples of 20 μl of each enzyme were assayed with casein at Ca^{2+} concentrations over the range 10 μM–3 mM. The derived K_d values were: ∇, wild-type m-80-k, 325 μM; $\square$, m-Bam-μ-Sal-m, 260 μM; and $\bigcirc$, m-Bam-μ-Fsp-m, 175 μM. The normalized means of duplicate data points were plotted and the lines shown were derived by fitting the results to the Hill equation as described previously [8].

The upper part of Figure 4(a) and the upper broken-line plot in Figure 4(b) show the K_d values for a set of hybrid calpains, all having m-calpain N-terminal residues 2–49, followed by a μ-type sequence extending stepwise to the C-terminus. The K_d value was decreased by the introduction of μ-calpain domains I–III, residues *59–498* (wild-type m-80, 325 μM; m-Bam-μ-Sal-m, 260 μM). The K_d value then decreased more steeply and progressively for each step throughout domain IV.

This pattern was maintained in the analogous series with the μ-type N-terminus (Figure 4a, lower part; Figure 4b, continuous-line plot). Introduction of the μ-type N-terminus, residues *2–58*, caused a decrease in K_d value to 250 μM for μ-Bam-m. There was then only a small reduction with the introduction of μ-calpain domains I–III (μ-Sal-m) and there was again a steep decrease with additional μ-type sequence in domain IV.

Not all constructs fell neatly into these patterns. Two hybrid calpains, m-Sal-μ-Fsp-m and m-Sal-μ-Dra-m, were well expressed and had heat-stabilities within the normal range. Their K_d values were unexpectedly high, namely 550 and 580 μM respectively.

Apart from these two exceptions, the range of K_d values observed in the two series extended from 325 μM for wild-type m-calpain to approx. 25 μM for wild-type μ-calpain. In terms of the stepwise reduction in K_d values displayed by hybrid calpains within this range, it may be concluded that the N-terminal region alone of 40–50 residues contributes approx. 25% of the difference in K_d value between μ- and m-calpain. In the presence of the m-calpain N-terminal peptide, the domain I–III sections of 360 residues between the *Bam*HI and *Sal*I sites also contributes approx. 20%, but the effects of the N-terminal region and of domains I–III are not additive. The linker peptide and domain IV, containing only 200 residues, contribute approx. 65% of the difference in K_d values.

DISCUSSION

Choice of mutations and characterization of the products

The μ- and m-calpains provide an interesting system for the study of Ca^{2+} binding, since their sequences are closely related (62% identical replacements, 29% conservative or semi-conservative replacements and only 9% non-conservative replacements), but they nonetheless have very different K_d values. In the C-terminal EF-hand-containing domains, which are the most likely source of the difference in K_d, the similarities are a little lower (52% identical, 34% conservative or semi-conservative replacements and 14% non-conservative).

There is a large literature concerning co-operative binding of Ca^{2+} to EF-hand proteins such as calmodulin, troponin C and

calbindin D_{9k} [23–28]. Many mutations have been described that affect Ca^{2+} binding in these model systems, but the results have often been unpredictable. In the present work, we attempted to explain the difference in K_d value between μ- and m-calpain, so the mutations were restricted to the conversion of m-type residues into the corresponding μ-type residues. The mutations were selected by comparison of the μ- and m-calpain sequences, together with inspection of the m-calpain structure.

Characteristics, such as expression level, activity and heat stability of most of the expressed mutant calpains, including both the point mutations and the hybrid calpains, were close to normal. This suggested that their structures had not been grossly disrupted by the mutations and that their K_d values could be meaningfully compared. The mutants have not been studied by crystallography, not least because this would be a demanding task, but mainly because the effects of these mutations on structure are likely to be of most interest in the presence of Ca^{2+}, conditions under which it is presently not possible to crystallize calpain.

Several mutations of EF-hand residues did not reduce K_d

The Ca^{2+}-co-ordinating loops of EF-hands 1–3 were first converted, one loop at a time, from the m-type into the μ-type. These mutations did not involve Ca^{2+}-co-ordinating residues. Some of the mutations, such as $Glu^{544} \rightarrow Asp$ or $Arg^{618} \rightarrow Lys$, are considered to be conservative and were less likely to affect Ca^{2+} binding. Others, such as $Glu^{586} \rightarrow Arg$ and $Lys^{595} \rightarrow Val$, were clearly non-conservative and were more likely to affect either the local intrinsic Ca^{2+} affinity or longer-range intramolecular interactions. However, the mutations in general caused only small increases in K_d, rather than the hypothetical decrease (Table 3), showing that the limited μ/m differences between the EF-hand loops are not by themselves responsible for the difference in K_d.

Some changes in salt-link and hydrophobic contacts did not reduce K_d

Two positions in domain IV were studied, where the salt links between remote residues appeared to be radically different between μ- and m-calpain. In the first case, an earlier report (Dr C. M. Hosfield, personal communication) has suggested that the linker arm between domain III and IV, residues 514–530, is important in the Ca^{2+} response of calpain. Residues 516–518 (Ile-Glu-Ala) form a short β-sheet backbone interaction with residues 636–638 (Phe-Lys-Leu), the latter residues being conserved in μ-calpain. In m-calpain, there is also a salt link between the side chains of Glu^{517} and Lys^{637}, which cannot be formed in μ-calpain where Glu^{517} is replaced by Gln^{528}. The μ-sequence also has an additional residue, Pro^{532}, in this region. The five m-calpain residues 517–521 (Glu-Ala-Asn-Ile-Glu) were therefore replaced with the corresponding six μ-calpain residues *528–533* (*Gln-Ala-Asn-Leu-Pro-Asp*). However, this did not significantly alter K_d values.

Secondly, the m-calpain residues 639–641 (Pro-Cys-Gln) were replaced with the corresponding μ-calpain residues *651–653* (*Asn-Lys-Lys*). These are highly non-conservative changes, which would be expected to cause a significant structural disturbance. Cys^{640} lies close to Asp^{513} (in the linker arm) and Gln^{641} makes contacts with the small subunit residues Thr^{71} and Gln^{179}. This mutation also had surprisingly little effect on K_d values.

Hydrophobic interactions are thought to be very important in regulating Ca^{2+} binding in EF-hand proteins [26]. The hydrophobic residues are very highly conserved between μ- and m-calpain, but one such mutation was made. In several mammalian m-calpains, Ile^{579} (in the E2 helix) is conserved and makes hydrophobic contacts to conserved hydrophobic residues in the F4 helix, including Val^{667} and Ile^{671}. Conversion of Ile^{579} to the corresponding μ-calpain residue Ser^{591}, in the expectation that this would weaken the hydrophobic interaction, caused only a small increase in K_d values.

Therefore it has not been possible so far to identify specific intramolecular interactions within domain IV of calpain that can explain the observed difference in K_d between values μ- and m-calpain.

Hybrid calpains indicate the relative contribution of segments of the large subunits to K_d

The idea that calpain might bind Ca^{2+} at positions outside domains IV and VI had been discussed for some time. Two Ca^{2+}-binding sites have recently been identified in domains I and II and they are clearly essential for calpain activity [7]. Ca^{2+} may also bind to the cluster of negative charges in the C2-like domain [29], although this has not yet been shown in an intact calpain. It is also clear that calpain activation must involve conformational changes throughout the entire calpain large subunit [5–7], implying that sections other than the C-terminal domain could influence K_d. Therefore several hybrid calpains were constructed to determine the relative contribution of the various regions of calpain to K_d values.

Comparison of the K_d values of wild-type m-80-k and μ-Bam-m (Figure 4) showed that the N-terminal region of the large subunit contributes 20–25% of the difference in K_d. This N-terminal region makes a number of contacts to the small subunit domain VI [5,6], and the release of these contacts is assumed to be an early step in activation, permitting autolysis of the large subunit N-terminal peptide, initially at Ala^9 and possibly later at Gly^{19} [3]. The N-terminus of μ-calpain is ten residues longer than that of m-calpain, so that its interactions cannot be modelled with confidence. But the results from the hybrid calpains show that the interaction of the μ-calpain N-terminus with domain VI is more easily disrupted in the presence of Ca^{2+} than the corresponding contact in m-calpain.

The pattern of K_d values in Figure 4 shows that domains I–III make relatively little contribution to K_d. This suggests that the Ca^{2+}-binding sites in domains I and II, although essential for activity, as well as the putative Ca^{2+}-binding sites in domain III, do not greatly influence the early steps of calpain activation.

Finally, Figure 4 shows that, within the C-terminal region of the large subunit, each subsection makes a significant contribution to the difference in K_d. The most marked decrease in the K_d value was caused by the introduction of the μ-calpain residues *499–581* (*Sal*I–*Fsp*I), which include the linker peptide between domains III and IV and EF-hand 1. This μ-type segment therefore includes and repeats the mutation of residues 517–521 described above, which by itself did not affect the K_d values. The K_d value of 110 μM for the μ-Fsp-m construct is consistent with a previous report [30] on fusion of human μ-calpain residues *1–544*, via a bridging Ser residue, to rat m-calpain residues 537–700, forming a hybrid with a K_d of approx. 50 μM (measured at low ionic strength) (Dr E. M. Vilei, personal communication).

Introduction of the μ-type residues *579–649* (*Fsp*I–*Dra*I), which contain EF-hands 2 and 3, caused a further decrease in K_d values. Eliminating Ca^{2+} binding at these two EF-hands had shown that they were of major importance for calpain activation [9], but the m-to-μ point mutations in these two EF-hands did not cause a decrease in the K_d values.

A molecular explanation for the decrease in K_d values resulting from interactions of the *499–581* (*Sal*I–*Fsp*I) section with the rest of the calpain molecule and similarly for the *579–649* section

(*Fsp*I–*Dra*I) cannot be provided at present. The results underline the fact that the Ca^{2+} requirement is dictated by relatively large-scale interactions.

Introduction of the final μ-type section of the large subunit, residues *650–726* (*Dra*I–C-terminus), caused a further reduction in K_d. EF-hands 4 and 5 do not bind to Ca^{2+}, but EF-hand 5 provides most of the hydrophobic surface required for heterodimer formation with domain VI in the small subunit. We speculate that subunit dissociation in μ-calpain occurs more easily than in m-calpain, but the difficulty in expressing calpain with a μ-type C-terminal region has prevented quantitative comparison of this interaction.

In conclusion, it has been shown within the large subunit of 700 residues that the N-terminal peptide region of 40–50 residues is responsible for 20–25 % of the difference in K_d values and that the C-terminal EF-hand-containing region of 200 residues accounts for 65 % of the difference. No single (or small set of) critical intramolecular interactions have so far been identified that can explain the difference in K_d values between μ- and m-calpain. It is probable that no such limited set exists and that the conformational change induced by Ca^{2+} in calpain involves co-operative large and small adjustments of perhaps hundreds of intramolecular contacts. Even in the model EF-hand-domain protein calbindin D_{9k}, in which co-operative binding of two Ca^{2+} atoms has been extensively studied, it was concluded 'that four-helix EF-hand domains should be treated as a single globally cooperative unit' [26]. This concept can clearly be extended to calpain, which has up to six EF-hands probably involved in Ca^{2+} binding within domains IV and VI, in addition to the recently established Ca^{2+}-binding sites in domains I and II [7] and potential binding sites in domain III.

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