Roles of individual EF-hands in the activation of m-calpain by calcium

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m-Calpain is a heterodimeric, cytosolic, thiol protease, which is activated by Ca²⁺-binding to EF-hands in the C-terminal domains of both subunits. There are four potential Ca²⁺-binding EF-hands in each subunit, but their relative affinities for Ca²⁺ are not known. In the present study mutations were made in both subunits to reduce the Ca²⁺-binding affinity at one or more EF-hands in one or both subunits. X-ray crystallography of some of the mutated small subunits showed that Ca²⁺ did not bind to the mutated EF-hands, but that its binding at other sites was not affected. The structures of the mutant small subunits in the presence of Ca²⁺ were otherwise identical to that of the Ca²⁺-bound wild-type small subunit. In the whole enzyme the wild-type macroscopic Ca²⁺ requirement (K_d) was approx. 350 μ M.

The mutations did not affect the maximum specific activity of the enzyme, but caused increases in K_d , which were characteristic of each site. All the EF-hands could be mutated in various combinations without loss of activity, but preservation of at least one wild-type EF-hand 3 sequence was required to maintain K_d values lower than 1 mM. The results suggest that all the EF-hands can contribute co-operatively to calpain activation, but that EF-hand 3, in both subunits, has the highest intrinsic affinity for Ca²⁺ and provides the major driving force for conformational change.

Key words: calcium binding, co-operativity, crystallography, Hill plot, site-directed mutagenesis.

INTRODUCTION

Calpains are heterodimeric, cytosolic, thiol proteases, which are regulated by Ca²⁺ binding at EF-hands in their C-terminal domains. Two principal isoforms, μ - and m-calpain, are expressed ubiquitously in mammals and have different *in vitro* Ca²⁺ requirements. For μ -calpain this requirement is in the range of 50 μ M Ca²⁺, and for m-calpain it is approx. 350 μ M [1–3]. These values are higher than the presumed values of intracellular Ca²⁺ concentration; additional factors including phospholipids and activator proteins appear to lower the Ca²⁺ requirement of the calpains *in vivo* [4–6]. Both μ - and m-calpain contain similar, but genetically distinct, large (80 kDa) catalytic subunits, comprising domains I–IV, and identical small (28 kDa) regulatory subunits, comprising domains V and VI.

The mechanism of Ca²⁺ regulation is of interest since calpain is an unusual protease in two respects: it is a heterodimer and it combines two EF-hand domains (domains IV and VI), one in each subunit and each containing several EF-hands, with a thiol protease function in the large subunit (domains I and II). Four EF-hand motifs were originally identified in the primary sequences of the C-terminal domain IV of both μ - and m-calpain large subunits [7], as well as in the C-terminal domain VI of the small subunit. A fifth EF-hand motif, slightly upstream of these four motifs, was detected in each of these domains in a more recent sequence comparison [8]. The amino acid sequences of the Ca²⁺-binding loops of EF-hands 2 and 3 conform excellently to the established canonical sequence [9], while those of EF-hands 1, 4 and 5 diverge increasingly from this sequence. Domain VI, when expressed alone in *Escherichia coli*, forms a homodimer in solution and in crystals [10]. Its structure has been solved in both the Ca²⁺-free and Ca²⁺-bound forms, revealing only a small Ca²⁺-induced change in conformation [11,12]. EF-hands 1, 2 and 3 in crystals of domain VI were found to be occupied by Ca²⁺ at

1 mM Ca²⁺, whereas EF-hand 4 was occupied by Ca²⁺ only at 20 mM Ca²⁺ [11]. The EF-hand 5 motif does not bind Ca²⁺, but is involved in the subunit interface. Since EF-hand 4 clearly binds Ca²⁺ with much lower affinity than the others, the work reported in the present study concentrated mainly on EF-hands 1–3.

It is well established that addition of Ca²⁺ to purified active calpain causes both aggregation and autolysis, which lead to complete loss of activity. When working with inactive mutants to prevent autolysis, addition of Ca²⁺ leads to aggregation, which can be seen as an increase in light scattering or as the formation of a visible precipitate [13]. It is assumed that these observations reflect a marked change in conformation of calpain in response to Ca²⁺, even though the difference in conformation of domain VI with and without Ca²⁺ is small [11]. It is also possible that the change in calpain conformation involves dissociation of the subunits, although this aspect is still debated [2,14,15].

The X-ray structure of m-calpain in the absence of Ca²⁺ has recently been described [16,17]. A Ca²⁺-bound structure is desirable for many purposes, such as revealing the conformational change associated with Ca²⁺ binding and demonstrating occupancy of EF-hands by Ca²⁺, but has not yet been obtained.

Due to the difficulties of aggregation and of crystallization in the presence of Ca²⁺, it does not appear, at present, to be feasible to obtain an accurate measure of Ca²⁺-binding stoichiometry in m-calpain, or to obtain direct evidence of Ca²⁺ occupation of specific EF-hands as a function of Ca²⁺ concentration. It was therefore necessary to use mutagenesis and changes in the apparent macroscopic K_d values to assess the relative contributions of individual EF-hands to the Ca²⁺ requirement of the enzyme. Critical bidentate ligating residues in EF-hand loops were mutated to reduce or eliminate Ca²⁺ binding at the altered sites, and the Ca²⁺ concentrations required for activity of the resultant enzymes were determined. The results suggested that all

Abbreviations used: m-80k, large 80 kDa subunit of rat m-calpain; 21k, 21 kDa domain VI of the rat calpain small subunit.

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the EF-hands act co-operatively in Ca^{2+} binding, but that EF-hand 3 provides a large proportion of the driving force for calpain activation.

MATERIALS AND METHODS

Calpain clones, enzyme expression and purification

The plasmid pET-24-m-80k-CHis₆, containing cDNA coding for the large 80 kDa subunit of rat m-calpain (m-80k) with a C-terminal His-tag, and the plasmids pET-20-21k and pACpET-21k, containing cDNA coding for the C-terminal 21 kDa domain VI of the rat calpain small subunit (21k), have been described previously [18,19]. The 21k construct represents, to a close approximation, the modified small subunit produced naturally in m-calpain by autolytic removal of domain V from the full-length small subunit, and it has been shown that the Ca^{2+} sensitivity of recombinant m-calpain is unchanged by loss of domain V [20]. For purposes of crystallography, the 21k constructs were expressed separately from pET-20-21k in *E. coli* [10]. Co-expression of large and small subunits in *E. coli*, and purification of the resultant heterodimeric enzymes, have also been described previously [18,21].

Site-directed mutagenesis

Single strand DNA was prepared from pET-24-m-80k-CHis₆ and from pET-20-21k for use as templates in the mutation of the large and small subunits, respectively. EF-hand mutations were generated by means of the Kunkel method [22], using the antisense oligonucleotides shown in Table 1. For EF-hands 1–3, the glutamic acid at position 12 of the Ca^{2+} binding loop was replaced by alanine. For EF-hand 4, which has an unusual system of Ca^{2+} co-ordination [11,12], the aspartic acid at position 11 was replaced by alanine. The mutations were confirmed by means of restriction digestion and DNA sequencing. The mutated 21k subunit cDNA was cloned, by means of *Xba*I/*Hind*III digestion, into pACpET for co-expression with the large subunit.

Nomenclature of mutant proteins

The mutated calpain subunits are described by abbreviations in which the mutated EF hands are shown in parentheses. For example, the term 21k(EF3) refers to the small subunit construct with a Glu-to-Ala mutation in EF-hand 3 (E195A). Similarly, the term m-80k(EF2/3) corresponds to the large subunit construct with the Glu-to-Ala mutation in both EF-hands 2 and 3 (E596A and E626A, respectively).

Table 1 Oligonucleotides used for site-directed mutagenesis of m-calpain EF-hands

Mutant	Mutation*	Antisense primer
21k(EF1)	E121A	5'-GGATATTATCATGAGAGCTGTAGCGCTG-3'
21k(EF2)	E174A	5'-GAGGTACTTGAAGGCTCAAAGCCC-3'
21k(EF3)	E195A	5'-TCAAAGGCCCGGGGAGTGCTTGTACCAATG-3'
21k(EF4)	D229A	5'-GCAGCTGATGAAGTTGCGGAAATCCATGTTCC-3'
80k(EF1)	E552A	5'-GATGGTCTCGAGTGCAAAGGCAGAGAT-3'
80k(EF2)	E596A	5'-GAGGATGTAGAAGGCTTCAGCCCCAG-3'
80k(EF3)	E626A	5'-GCCTTCGCGCATAGCATATGAATTCATGG-3'
80k(EF4)	D660A	5'-CGGCACAAAGTTGGCAAATCGATGATGAGTTC-3'

* For the small subunit, the residue numbers refer to the full-length rat calpain small subunit of 268 residues, although the 21k protein contains only the C-terminal 184 residues.

Crystallography of 21k mutants in the presence of Ca^{2+}

Attempts were made to crystallize most of the small subunit mutants described in this study. Crystals were obtained in the presence of 50–250 mM Ca^{2+} , 6–13 % poly(ethylene glycol) 8000 as a precipitant, 50 mM cacodylate (pH 6.6) and 7–13 % (v/v) glycerol, conditions very similar to those used for the wild-type 21k [11]. X-ray diffraction data were collected on the RAXIS-IIC system at cryogenic temperatures (100 K) using an appropriate cryoprotectant. It should be noted that the conditions required for crystallization of the small subunit differ from those used for titration of the Ca^{2+} requirement of the whole enzyme.

Titration of the Ca^{2+} requirement of active m-calpain mutants for casein hydrolysis

Samples of mutant or wild-type heterodimeric m-calpains were dialysed overnight at 4 °C against 50 mM Tris/HCl (pH 7.6), 50 mM NaCl, 2 mM EDTA and 10 mM 2-mercaptoethanol. Protein concentrations were adjusted to provide 5–7 units of activity (3–4 µg of pure calpain) in a 20 µl aliquot. Titrations of the Ca^{2+} requirement for casein hydrolysis were performed as described previously, in 50 mM Tris/HCl (pH 7.6), 0.2 M NaCl and 10 mM 2-mercaptoethanol (buffer A) [23].

The activity data were normalized by expression as the fraction of maximum activity, and were fitted to the Hill equation:

$$y = x^h / (K_d^h + x^h)$$

where y is the fraction of maximum activity, x is $[\text{Ca}^{2+}]$, and h is the Hill coefficient. K_d is the apparent macroscopic dissociation constant of the whole enzyme for the Ca^{2+} /enzyme binding equilibrium, and is equal to $[\text{Ca}^{2+}]_{0.5}$, the value of $[\text{Ca}^{2+}]$ at which half-maximum activity is observed. K_d is also defined in the present study as the ' Ca^{2+} requirement' of the enzyme.

Statistics

Within one titration, the duplicate data points at each value of $[\text{Ca}^{2+}]$ were within 3 % of each other. Non-linear regression to the Hill equation gave K_d values with standard errors of 5 % and h values with standard errors of 5–10 %. As explained previously [23], it is not feasible to titrate more than three different enzymes at any one time. However, all experiments included a control sample in the same K_d range as the experimental sample, in order to detect large disparities over several months. Consequently the wild-type 21k/m-80k was titrated at least eight times, most mutants were titrated three times, and a few were titrated only once or twice. The statistical significance of differences in K_d values in Table 3 was assessed by means of an unpaired t test.

Measurement of Ca^{2+} - and Tb^{3+} -induced aggregation

Aggregation was monitored by continuous measurement of right-angle light scattering in a Perkin Elmer LS50 fluorimeter set for excitation and emission at 340 nm, with filter cut-off at 290 nm, and slit widths of 2.5 nm [13]. Inactive 21k/C105S-m-80k calpain (100 µg/ml, 1 µM), was stirred continuously in buffer A at 22 °C. CaCl_2 or TbCl_3 was added at intervals of 2–3 min in small aliquots from 1 M stock solutions.

RESULTS

Crystallography of domain VI mutants

Previous crystallography of wild-type 21k (domain VI) had shown that EF-hands 1–3 were occupied at 1 mM Ca^{2+} , EF-hand

Figure 1 Electron densities calculated from the X-ray diffraction data for mutants 21k(EF1) and 21k(EF3), crystallized in the presence of Ca²⁺

(A) shows EF-hand 1 in 21k(EF1) with the E121A mutation, and free of Ca²⁺, (B) shows the unaltered EF-hand 3 in the same protein, 21k(EF1), containing Ca²⁺, (C) shows the unaltered EF-hand 1 in 21k(EF3), containing Ca²⁺ and (D) shows EF-hand 3 with the E195A mutation in the same protein, 21k(EF3), free of Ca²⁺.

4 was occupied only at 20 mM Ca²⁺, and EF-hand 5 did not bind Ca²⁺ [11,12]. Only 21k(EF1), 21k(EF2) and 21k(EF3) gave single crystals suitable for X-ray diffraction, although their quality was significantly poorer than that of the wild-type protein, and the crystals diffracted to a resolution varying between 2.5 Å and 3.3 Å. They all gave rise to multiple crystal forms in the presence of Ca²⁺, and the structures were individually refined for each mutant and each crystal form. The 21k(EF1) mutant crystallized in P6₅, C2, and P212121 space groups, 21k(EF2) in the P6 space group and 21k(EF3) in the C2221 space group. Their structures were solved by molecular replacement, using the co-ordinates of one of the two monomers of the high-Ca²⁺ structure (protein data-base code 1dvi) [11]. In all of the crystals of the EF-hand mutants described in the present study, the asymmetric unit contained a homodimer, as observed previously for wild-type 21k [10]. The

structures were refined to values of the crystallographic R-factor ranging from 0.20–0.23 and R-free from 0.28–0.35. Attempts to solve these structures using the co-ordinates of the apo form were unsuccessful, giving an early indication that the EF-hand mutants do not adopt the apo conformation in the presence of Ca²⁺. The single 21k(EF4), double [21k(EF1/2, EF1/3 etc.)], triple [21k(EF1/2/3)] and quadruple [21k(EF1/2/3/4)] mutants were prone to aggregation and/or gave severely twinned crystals.

In each case where the structure could be solved, the Ca²⁺ ion was absent from the mutated EF-hand, whereas the remaining EF-hands in the same protein contained Ca²⁺. In Figure 1 the two upper diagrams (A and B) show that in the 21k(EF1) protein the mutated EF-hand 1 (E121A) is free of Ca²⁺, and that the unaltered EF-hand 3 (Glu¹⁹⁵) is occupied by Ca²⁺. Conversely the two lower diagrams (C and D) show that in the 21k(EF3)

protein the unaltered EF-hand 1 (Glu¹²¹) is occupied by Ca²⁺, while the mutated EF-hand 3 (E195A) is free of Ca²⁺. In all other regions except for the mutated EF-hands, the structures of these small subunit proteins were the same as that of the Ca²⁺-bound form of the wild-type 21k. Binding of Ca²⁺ at any subset of sites in the small subunit therefore induces essentially the same conformational change that leads to the same final Ca²⁺-bound form of the subunit. This co-operativity was shown most explicitly in 21k(EF1), by the fact that when EF-hands 2 and 3 were occupied by Ca²⁺, the mutated loop of EF-hand 1, although free of Ca²⁺, was well-defined in the electron density map and in the Ca²⁺-bound conformation. Consequently, the root-mean-square deviation between the 21k(EF1) and wild-type 21k structures, both in the presence of Ca²⁺, was 0.50 Å, including the loop in EF-hand 1. In 21k(EF2) and 21k(EF3), the loops containing mutations did not contain Ca²⁺ and were less well-defined in the electron density map than the corresponding loops in the wild-type protein, so that the root-mean-square deviation between mutated and wild-type structures was 0.48 Å, except for the mutated loops. The disorder in these loops is assumed to result from loss of hydrogen-bonding caused by replacement of the glutamic acid carboxyl group side chain by the methyl group of alanine. These results demonstrated that the mutations abolished Ca²⁺ binding at the mutated sites without significantly affecting the global protein conformation, and did not interfere with Ca²⁺ binding at the unaltered EF-hands.

Effects of Ca²⁺ and Tb³⁺ on calpain aggregation

Addition of Ca²⁺ to 21k/C105S-m-80k (100 µg/ml, 1 µM) in buffer A caused an increase in right-angle light scattering with time, as reported previously for this and other inactive mutants [13]. The effect was readily observed at 250 µM Ca²⁺, and the increase was faster at higher Ca²⁺ concentrations, but no visible precipitate appeared up to 1.5 mM Ca²⁺. This does not appear to interfere with the normal calpain assay, in which the calpain concentration is < 0.1 µM, autolysis accompanies the reaction and the mixture includes 4 mg/ml casein. In contrast, precipitation of calpain was already visible at 250 µM Tb³⁺. It was also observed that Tb³⁺ at > 1 mM caused rapid precipitation of a solution of 4 mg/ml casein in buffer A. A normal assay of a 5 µg sample of 21k/m-80k, carried out to assess the importance of these problems, showed a maximum activity of 7.3 units at 5 mM Ca²⁺, an apparent maximum activity of 5 units at 2.4 mM Tb³⁺, and even lower activity at a higher concentration of Tb³⁺. The conventional casein assay is clearly unreliable in the presence of Tb³⁺, and there are serious obstacles to the use of Tb³⁺ to measure binding stoichiometry in calpain [24,25].

Enzymic activity of mutant heterodimeric calpains

The mutant heterodimeric calpains were well expressed and could be purified by established procedures. They behaved normally on casein zymography, which involves non-denaturing gel electrophoresis (results not shown) [26,27]. Their specific activities at saturating Ca²⁺ concentrations were in the range of 1400–1900 units/mg of protein, as reported for the wild-type enzyme [23], and there was no correlation between variations within this range and Ca²⁺ requirement. It is concluded that the EF-hand mutations affected K_d , but did not affect the catalytic efficiency of the enzymes.

Ca²⁺ binding at individual EF-hands

The phrase 'reduction in Ca²⁺ binding' is used here for brevity. The mutation of Glu to Ala at position 12 of the EF-hand

Table 2 Ca²⁺ requirement (K_d) and Hill coefficient (h) of m-calpains with EF-hand mutations

(A) single mutation in one or both subunits, (B) two or more mutations in one subunit and (C) mutations involving EF-hand 4. K_d values are represented as means ± S.E.M., n is indicated in parentheses. In two cases the K_d value represents a single measurement. N.D., not determined.

Small subunit mutation	Large subunit mutation	K_d (µM)	h	Sites*
A				
wild-type	wild-type	367 ± 6 (8)	5.0	6
wild-type	EF1	348 ± 2 (2)	3.6	5
wild-type	EF2	472 ± 24 (3)	5.6	5
wild-type	EF3	830 ± 18 (3)	4.5	5
EF3	wild-type	675 ± 19 (3)	5.0	5
EF3	EF3	1699 ± 46 (4)	4.5	4
B				
wild-type	EF1/2	362 ± 36 (2)	4.2	4
wild-type	EF1/3	896 ± 35 (3)	4.2	4
wild-type	EF2/3	1062 ± 45 (4)	4.5	4
wild-type	EF1/2/3	946 ± 61 (3)	4.5	3
EF1/2/3	wild-type	930 ± 38 (2)	3.1	3
EF1/2/3	EF1/2/3	7410 (1)	3.1	0
C				
EF1/3/4	wild-type	769 ± 20 (2)	3.2	4
EF1/2/4	EF1/2	822 ± 30 (2)	2.4	2
EF1/2/4	wild-type	542 ± 2 (2)	2.5	4
EF2/3/4	wild-type	844 ± 43 (3)	2.4	4
EF1/2/3/4	wild-type	897 (1)	2.6	3
EF1/2/3/4	EF1/2/3/4	N.D.†		

* This column represents the presumed number of functional EF-hands in the given calpain. The two EF-hand 4 motifs have not been included in this estimate, since they probably do not bind Ca²⁺ in most cases.

† K_d was not determined, but low activity was detected on casein zymograms at 5 mM and 20 mM Ca²⁺.

loop has been used frequently, and has been found in other cases to reduce the Ca²⁺ affinity of the site approx. 100-fold [28–31]. The X-ray data of isolated small subunits crystallized in the presence of 50–250 mM Ca²⁺ showed that this mutation abolished Ca²⁺ binding at the mutated sites. It was therefore likely that Ca²⁺ binding at the mutated sites in the whole enzyme had also been abolished, at least in the range of 0.1–2.0 mM Ca²⁺ used in most of the titrations. Domains IV and VI are virtually identical in the crystal structure of m-calpain [16,17], and have approx. 60% sequence identity. It therefore seems reasonable to assume that the behaviour of EF-hand mutations in the large subunit would be very similar to that shown in the small subunit. This was supported by the patterns of K_d values described below, but the Ca²⁺-binding stoichiometry in the mutant calpains has not been established by an independent method.

Ca²⁺ titration data resulting from all the EF-hand mutations are given in Table 2, in three sections. Table 2(A) lists the K_d values for single mutations that caused reduction in Ca²⁺ binding at only one position in a given subunit. These results show that a reduction in Ca²⁺ binding at EF-hand 1 in the large subunit had no significant effect on K_d , while reduction in Ca²⁺ binding at EF-hand 2 increased the K_d slightly, and at EF-hand 3 produced a major increase in K_d .

Conversely, instead of describing increases in K_d caused by mutation, we have found it easier to understand the data in terms of sites which have not been altered. Table 2(B) shows the K_d values for some calpains with two or three mutations in only one subunit, while the other subunit remained as wild-type. With three mutations (in either one of the two subunits), the K_d values

Table 3 Change in K_d resulting from introduction of particular EF-hand mutations, in the presence of other mutations

The changes in K_d represent the mean K_d value of mutant no. 2 minus the mean K_d value of mutant no. 1. The P values indicate the significance of these differences as determined by unpaired t tests; N.S., not significant; N.D., not determined, where insufficient data were available. wt, wild-type.

Affected EF-hand	Pair-wise comparison		Change in K_d (μ M)	P value
	Mutant no. 1	Mutant no. 2		
m-80k EF-hand 1	wt	21k/m-80k(EF1)	+3	N.S.
	21k/m-80k(EF2)	21k/m-80k(EF1/2)	-110	0.044
	21k/m-80k(EF3)	21k/m-80k(EF1/3)	+66	0.16
	21k/m-80k(EF2/3)	21k/m-80k(EF1/2/3)	-115	N.S.
21k EF-hand 1	21k(EF2/3/4)/m-80k	21k(EF1/2/3/4)/m-80k	+43	N.D.
m-80k EF-hand 2	wt	21k/m-80k(EF2)	+108	0.013
	21k/m-80k(EF1)	21k/m-80k(EF1/2)	+14	N.S.
	21k/m-80k(EF3)	21k/m-80k(EF2/3)	+232	0.009
	21k/m-80k(EF1/3)	21k/m-80k(EF1/2/3)	+50	N.S.
21k EF-hand 2	21k(EF1/3/4)/m-80k	21k(EF1/2/3/4)/m-80k	+128	N.D.
m-80k EF-hand 3	wt	21k/m-80k(EF3)	+466	0.0001
	21k/m-80k(EF1)	21k/m-80k(EF1/3)	+548	0.0001
	21k/m-80k(EF2)	21k/m-80k(EF2/3)	+590	0.0001
	21k/m-80k(EF1/2)	21k/m-80k(EF1/2/3)	+584	0.001
21k EF-hand 3	wt	21k(EF3)/m-80k	+311	0.0001
	21k(EF1/2/4)/m-80k	21k(EF1/2/3/4)/m-80k	+355	N.D.

were approx. 940 μ M. With two mutations in the large subunit, when only EF-hand 1 was unaltered and able to bind Ca^{2+} significantly [21k/m-80k(EF2/3)], the K_d was 1.11 mM. When only EF-hand 2 was unaltered [21k/m-80k(EF1/3)] the K_d value was 896 μ M and when only EF-hand 3 was unaltered [21k/m-80k(EF1/2)] the K_d value was 362 μ M, unchanged from the wild-type value.

The observation that both the triple mutants 21k(EF1/2/3)/m-80k and 21k/m-80k(EF1/2/3) had K_d values of 940 μ M (Table 2B) also demonstrated that Ca^{2+} binding in either of the subunits alone is sufficient to activate calpain, and that no single wild-type EF-hand is essential for activation.

Several other mutant calpains were prepared, with different sets of multiple EF-hand mutations including EF-hand 4 in one or both subunits (Table 2C). The results indicate that mutation of EF-hand 4 had little effect on K_d values. This is consistent with the X-ray results which had shown that EF-hand 4 was occupied only at very high $[\text{Ca}^{2+}]$. Almost all the mutants described had K_d values less than 1 mM, and only when EF-hand 3 in both subunits had been mutated, in 21k(EF3)/m-80k(EF3) or in 21k(EF1/2/3)/m-80k(EF1/2/3), did K_d increase, to 1.7 mM and 7.4 mM, respectively. This underlines again the importance of EF-hand 3. The octuple 21k(EF1/2/3/4)/m-80k(1/2/3/4) mutant, although it had not been purified, showed a trace of activity on casein zymograms in the presence of 5 or 20 mM Ca^{2+} . In mutants such as 21k(EF1/2/3)/m-80k(EF1/2/3) and 21k(EF1/2/3/4)/m-80k(1/2/3/4), it appears likely that activation is due to Ca^{2+} binding with much lower affinity at some of the mutated sites or at EF-hand 4, and possibly elsewhere in the molecule.

To summarize these findings, it seems likely that Ca^{2+} binding at any one EF-hand is sufficient to activate the enzyme. In the wild-type enzyme EF-hand 3 is most important for activation, EF-hand 2 may make a lesser contribution to activation and binding at EF-hand 1 appears to be unimportant for enzyme activation.

Co-operativity and values of the Hill coefficient

In attempting to assess the co-operativity of Ca^{2+} binding, it was useful to make pair-wise comparisons between K_d values. This made it possible to assess the effect of a given mutation in the context of different background sets of normal and mutated sites. Since it is difficult to pick these pairs out of Table 2, the differences between pairs are listed in Table 3. They show that mutation of EF-hand 1, in either subunit, gave small effects on K_d (changes in K_d from -115 μ M to +66 μ M). Mutation of EF-hand 2 raised the K_d by approx. 130 μ M (changes in K_d from 14–232 μ M), and mutation of EF-hand 3 caused a consistently larger increase in K_d (changes in K_d of approx. 330 μ M in the small subunit and 466–590 μ M in the large subunit). If any one mutation were particularly disruptive, it would be expected to have a much greater effect on additional mutations, but this was not observed. These results indicate that the co-operativity of Ca^{2+} binding, both within each subunit and between subunits, is a property of the whole calpain molecule, and is not disrupted by any of the EF-hand mutations used here.

The Hill coefficients derived from curve-fitting are shown in Table 2. For most mutants, the Hill coefficients fell in the range 4.2–5.6, but for small subunit multiple mutants the Hill coefficients were between 2.4 and 3.1. There was no correlation between the Hill coefficient of a given construct, and its presumed number of functional Ca^{2+} binding sites.

DISCUSSION

m-Calpain activity is obviously dependent on Ca^{2+} binding to EF-hands in the C-terminal domains (IV and VI) of both the large and small subunits, but the relative contributions of the EF-hands to the Ca^{2+} response of the enzyme were not known. We have therefore generated a series of EF-hand mutations in both subunits, which were expected to reduce the affinity of Ca^{2+} binding by approx. 100-fold at the given positions [28–31]. The Ca^{2+} titrations were performed with pure enzymes under standardized *in vitro* conditions, in order to estimate the contributions of individual EF-hands [23].

Crystallography of 21k mutants

X-ray crystallography of the 21k single EF-hand mutants demonstrated that Ca^{2+} binding at any position in the small subunit causes a change to the same final conformation. This conformation is identical to that of the wild-type domain VI in the presence of Ca^{2+} , except that the mutated EF-hands were free of Ca^{2+} . It is assumed that the changes in domain VI, and similar changes in domain IV, must form the basic trigger for calpain activation [16,17].

Ca^{2+} titrations in the whole enzyme and comparisons of K_d values

The normal expression, purification, and specific activities of the mutant whole enzymes demonstrated that the mutations had not disturbed the global structure and function of the enzymes.

Measurement of Ca^{2+} -binding stoichiometry in m-calpain would be very useful, but does not appear to be feasible at present. Recent extensive efforts to measure Ca^{2+} binding in m-calpain gave values that depended on the method used, and were usually larger than the number of EF-hands (Dr D. E. Goll, personal communication). Ca^{2+} binding to recombinant forms of domains IV and VI of calpain has been measured previously,

using a filter assay, showing approximately two binding sites in the small subunit with a K_d of 150 μM [32]. It has been reported, using equilibrium dialysis, that μ -calpain binds 8 mol of Ca^{2+} /mol of enzyme, independently and with a K_d of 25 μM [33]. This method cannot be used for m-calpain, both because of aggregation and also because m-calpain and its mutants have a much higher range of K_d than μ -calpain. Measurement of conformational change as a function of $[\text{Ca}^{2+}]$ by means of trinitrobenzene sulphonate fluorescence, which has been used with some calpain fragments [34], has not been successful with the whole enzyme. Terbium chloride is frequently used to study Ca^{2+} -binding sites, because of the characteristic enhancement of Tb^{3+} fluorescence when bound to protein, and in favourable cases binding at specific sites can be monitored by tyrosine- or tryptophan-sensitized Tb^{3+} luminescence [35,36]. It has been shown previously that Tb^{3+} can activate both μ - and m-calpain, but it also causes calpain aggregation [24,25]. We briefly repeated some of these Tb^{3+} experiments, and confirmed that Tb^{3+} cannot be used for precise measurements with m-calpain.

It was therefore necessary to rely on K_d values to assess the effects of EF-hand mutations. The K_d values are apparent macroscopic dissociation constants, reflecting both the affinity of the remaining functional EF-hands as well as co-operative interactions between them. In m-calpain, where it appears likely that up to six EF-hands bind Ca^{2+} in the given conditions, the data do not permit determination of microscopic Ca^{2+} dissociation constants for each site. However, Table 3 shows that the effects on K_d of introducing mutations at each EF-hand were fairly consistent, regardless of which other EF-hands were still functional, implying that the mutations primarily affected the Ca^{2+} affinity of each site.

From all of these results it was concluded that the affinities of the individual wild-type EF-hands for Ca^{2+} and their contribution to activation of the enzyme decrease in the order: EF-hand 3 $\gg$ EF-hand 2 $>$ EF-hand 1 $\gg$ EF-hand 4. It is interesting to note that the Ca^{2+} binding coils of EF-hands 2 and 3 are both in excellent agreement with the consensus sequence for this motif, but differ very greatly in their affinity for Ca^{2+} [37].

In studies of the EF-hands in calmodulin, it has been shown that target peptides, which bind calmodulin between the EF-hand domains, increase the Ca^{2+} -binding affinity of the mutated EF-hands [28]. The presence of calpain substrates or other binding factors may similarly affect the affinities of the calpain EF-hands, which could explain the reduction in K_d caused by phospholipids and calpain activator proteins [4–6]. These factors are probably important *in vivo*, but do not affect our comparisons of mutated calpains, which were conducted with highly purified enzymes in identical incubation conditions.

Importance of specific EF-hands for co-operativity

In EF-hand proteins, such as calmodulin, troponin C and S-modulin, Ca^{2+} binding induces specific movements in EF-hands which are responsible for generating global conformational changes [28–31]. Regulation of these proteins can therefore be altered by blocking the binding of Ca^{2+} at critical binding site(s). For example, a Glu-to-Ala mutation at position 12 of EF-hand 1 in skeletal muscle troponin C prevented Ca^{2+} -induced conformational opening of the protein, even when Ca^{2+} bound to the mutated site with very low affinity [30]. In the same way, reduction in Ca^{2+} binding at EF-hand 3 in S-modulin abolished function, even though a functional EF-hand 2 remained [31]. In m-calpain, however, it has been shown that enzymic activity is not absolutely dependent on wild-type Ca^{2+} binding at any individual EF-hand, or in either subunit, since activation by Ca^{2+}

occurred in the presence of all tested combinations of EF-hand mutations. It is therefore clear that all of the EF-hands in both subunits of calpain are linked in driving conformational change by a co-operative mechanism. Co-operativity is traditionally assumed to give rise to values of the Hill coefficient, h , greater than 1 [38,39]. The h values for calpains composed of large subunit mutants co-expressed with the wild-type 21k were between 4.2 and 5, very similar to that of the wild-type enzyme. For calpains consisting of small subunit triple mutants co-expressed with any form of the large subunit, the h values were between 2.4 and 3.2. We do not presently understand the significance of these values, which show no relationship to the expected number of binding sites, and the values should be interpreted with caution. As recently reviewed, the value of h for independent binding at multiple sites should remain below 2, but if a ligand-induced conformational change to an activated-state occurs, as in calpain, the value of h may approach the number of functional binding sites without assuming co-operativity [39]. The X-ray crystallography data and the patterns of K_d and h values strongly support a co-operative mechanism for the response of m-calpain to Ca^{2+} binding, but do not strictly prove it. In conclusion, no single unaltered Ca^{2+} binding site is required for activation of the enzyme, and the enzyme can still be activated by sufficiently high levels of Ca^{2+} when all EF-hands have been mutated. The K_d values for the various mutant calpains suggest that EF-hand 4 is unlikely to be involved, and that EF-hands 2 and 3, in both subunits of m-calpain, act co-operatively in binding Ca^{2+} and activating calpain. The major driving force for conformational change and calpain activation clearly derives from Ca^{2+} binding at EF-hand 3 in both subunits.

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