The effects of truncations of the small subunit on m-calpain activity and heterodimer formation

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In order to study subunit interactions in calpain, the effects of small subunit truncations on m-calpain activity and heterodimer formation have been measured. It has been shown previously that active calpain is formed by co-expression of the large subunit (80 kDa) of rat m-calpain with a Δ86 form (21 kDa) of the small subunit. cDNA for the full-length 270 amino acid (28.5 kDa) rat calpain small subunit has now been cloned, both with and without an N-terminal histidine tag ($NHis_{10}$). The full-length small subunit constructs yielded active calpains on co-expression with the large subunit, and the small subunit was autolysed to 20 kDa on exposure of these calpains to Ca^{2+}. A series of deletion mutants of the small subunit, $NHis_{10}$-Δ86, -Δ99, -Δ107, and -Δ116, gave active heterodimeric calpains with unchanged specific activities, although in decreasing yield, and with a progressive decrease in stability. $NHis_{10}$-Δ125 formed a heterodimer which was inactive and unstable. Removal of 25 C-terminal residues from Δ86, leaving residues 87–245, abolished both activity and heterodimer formation. The results show that: (a) generation of active m-calpain in *Escherichia coli* requires heterodimer formation; (b) small subunit residues between 94 and 116 contribute to the stability of the active heterodimer but do not directly affect the catalytic mechanism; (c) residues in the region 245–270 are essential for subunit binding. Finally, it was shown that an inactive mutant $Cys^{105} \rightarrow$ Ser-80k/Δ86 calpain, used in order to preclude autolysis, did not dissociate in the presence of Ca^{2+}, a result which does not support the proposal that Ca^{2+}-induced dissociation is involved in calpain activation.

INTRODUCTION

The calpains (EC 3.4.22.17) are cytoplasmic Ca^{2+}-dependent cysteine proteinases consisting of an ~ 80 kDa catalytic subunit (the large subunit) and a ~ 30 kDa subunit, which is usually described as regulatory (the small subunit) [1,2]. The large subunits consist of four domains (I–IV) and the small subunits consist of two domains (V,VI) [3,4]. The active site residues are located in domain II, and the C-terminal domains (IV and VI) of both subunits contain putative E-F hand sequences, some of which bind Ca^{2+} [5–8]. Binding of Ca^{2+} to the C-terminal domains is thought to cause conformational changes in the whole enzyme, which increase the accessibility or reactivity of the active site. This leads both to substrate hydrolysis and to rapid autolysis, which involves removal of domain V of the small subunit and N-terminal cleavage of the large subunit [9,10]. We have recently clarified some aspects of the relationships between autolysis, enzyme activation and Ca^{2+}-dependence in m-calpain [10a].

Calpain, when isolated in the presence of EDTA according to usual procedures, is a heterodimer, and the subunits can be separated using only mild denaturants such as 1 M sodium thiocyanate [11]. It has been proposed recently, however, that Ca^{2+} causes reversible dissociation of the two subunits as part of the normal activation mechanism [12,13]. This is an important point, but one which is very difficult to confirm, since studies of calpain in the presence of Ca^{2+} are complicated by autolysis and aggregation.

Some results relevant to subunit interactions have been reported. The small subunit presumably makes many contacts with the large subunit, including at least one with the active site in domain II when the small subunit is autolysed. At least one subunit binding site is located within the C-terminal domain of each subunit, since fragments corresponding to domains IV and VI, recovered after prolonged autolysis, remain bound to each other, whereas a fragment comprising most of domains I and II of the large subunit was not bound to other fragments [9,14]. Treatment with carboxypeptidase showed that one or two C-terminal residues of both subunits could be removed without effect, but that the last eight to ten C-terminal residues of both subunits were important for calpain activity [15]. It was then shown that removal of 22 C-terminal residues from the small subunit abolished its ability to reconstitute active calpain [6]. This result strongly suggested that formation of the heterodimer was essential for activity.

Bacterial co-expression of the rat m-calpain 80 kDa large subunit with a 21 kDa fragment of the rat calpain small subunit (now referred to as Δ86, see below) generates a fully active heterodimeric calpain [16,17]. This result has provided a convenient system for testing the effects of mutations in either of the subunits. In the present work, we have sought to determine the minimum sequence requirements for binding of the small subunit to the large subunit, and to establish whether the presence of a small subunit is essential for calpain activity.

EXPERIMENTAL

Escherichia coli strain BL21(DE3), and the plasmids pET-16b(+), pET-20b(+) and pET-24d(+) were obtained from Novagen Inc. (Madison, WI, U.S.A.). The plasmid pACpET-24, which is compatible in *E. coli* with the pET vectors, has been described previously [17].

Abbreviations used: 2-ME, 2-mercaptoethanol; 80k-$CHis_6$, pET-24d-80k-$CHis_6$ construct; $NHis_{10}$, N-terminal histidine tag; $Cys^{105} \rightarrow$ Ser-80k-$CHis_6$, active site $Cys^{105} \rightarrow$ Ser mutation in the large subunit.

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CAT
NdeI
ATG TTC TTG GTG AAC TCG TTC TTG AAG GGC GGC GGT GGC GGT GGG GGA GGC GGG GGT CTG  60
Met Phe Leu Val Asn Ser Phe Leu Lys Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Leu  20

GGC GGG GGC CTG GGC AAT GTG CTC GGA GGC CTG ATC AGC GGA GCT GCT GGA GGC GGC GGC 120
Gly Gly Gly Leu Gly Asn Val Leu Gly Gly Leu Ile Ser Gly Ala Ala Gly Gly Gly Gly  40

GGT GGA GGC GGC GGT GGC GGC ATG GGA CTG GGA GGA GGT GGC GGA GGC GGT GGA ACA GCC 180
Gly Gly Gly Gly Gly Gly Gly Met Gly Leu Gly Gly Gly Gly Gly Gly Gly Gly Thr Ala  60

ATG CGT ATC CTG GGC GGA GTC ATT AGC GCC ATC AGC GAG GCT GCT GCG CAG TAC AAC CCG 240
Met Arg Ile Leu Gly Gly Val Ile Ser Ala Ile Ser Glu Ala Ala Ala Gln Tyr Asn Pro  80

GAG CCC CCG CCC CCA CGT AGC CAT TAC TCT AAT ATT GAG GCC AAC GAG AGT GAA GAG GAA 300
Glu Pro Pro Pro Pro Arg Ser His Tyr Ser Asn Ile Glu Ala Asn Glu Ser Glu Glu Glu 100

CGT CAG TTC AGG AAA CTT TTT GTC CAG CTG GCT GGA GAC GAC ATG GAG GTC AGC GCT ACA 360
Arg Gln Phe Arg Lys Leu Phe Val Gln Leu Ala Gly Asp Asp Met Glu Val Ser Ala Thr 120

GAA CTC ATG AAT ATC CTC AAC AAG GTC GTG ACC CGA CAC CCA GAT CTG AAA ACT GAT GGG 420
Glu Leu Met Asn Ile Leu Asn Lys Val Val Thr Arg His Pro Asp Leu Lys Thr Asp Gly 140

TTC GGC ATT GAC ACT TGT CGG AGC ATG GTG GCT GTT ATG GAT AGT GAC ACC ACA GGC AAG 480
Phe Gly Ile Asp Thr Cys Arg Ser Met Val Ala Val Met Asp Ser Asp Thr Thr Gly Lys 160

CTG GGC TTT GAG GAG TTC AAG TAC CTC TGG AAC AAC ATT AAA AAG TGG CAG GGT ATA TAC 540
Leu Gly Phe Glu Glu Phe Lys Tyr Leu Trp Asn Asn Ile Lys Lys Trp Gln Gly Ile Tyr 180

AAA CGC TTT GAT ACT GAC CGA TCA GGG ACC ATT GGT AGC AAT GAA CTC CCA GGG GCC TTT 600
Lys Arg Phe Asp Thr Asp Arg Ser Gly Thr Ile Gly Ser Asn Glu Leu Pro Gly Ala Phe 200

GAG GCA GCA GGA TTC CAC CTG AAT CAG CAT ATC TAC AGC ATG ATC ATC CGC CGC TAC TCG 660
Glu Ala Ala Gly Phe His Leu Asn Gln His Ile Tyr Ser Met Ile Ile Arg Arg Tyr Ser 220

GAC GAG ACC GGG AAC ATG GAT TTC GAT AAC TTC ATC AGC TGC TTG GTC AGG CTG GAT GCC 720
Asp Glu Thr Gly Asn Met Asp Phe Asp Asn Phe Ile Ser Cys Leu Val Arg Leu Asp Ala 240

ATG TTC CGT GCT TTC AGA TCT CTT GAC AAA AAT GGC ACT GGA CAG ATC CAA GTG AAC ATC 780
Met Phe Arg Ala Phe Arg Ser Leu Asp Lys Asn Gly Thr Gly Gln Ile Gln Val Asn Ile 260

CAG GAG TGG TTG CAG CTG ACT ATG TAT TCC TGA GTGGAAGTCACAGACCTGCCGCCTCACTGCTCACT 840
Gln Glu Trp Leu Gln Leu Thr Met Tyr Ser  *                                      270

GTGAAGCTT
   HindIII
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Figure 1 cDNA and deduced amino-acid sequence of the small subunit of rat calpain

This clone was obtained from total rat lung RNA by means of reverse transcription PCR with the primers described in the Experimental section, which introduced *Nde*I and *Hin*dIII sites for use in cloning into the expression vectors. The four commonly described putative Ca^{2+}-binding loops are shown with double underlining, and a fifth one [8] is indicated with a single underline.

Calpain large subunit

Cloning of the cDNA for the rat m-calpain 80 kDa subunit (strictly 79880 Da, here referred to as 80k) to form the construct pET-24d-80k, the addition of the C-terminal His-tag to form the construct pET-24d-80k-$CHis_6$ (80k-$CHis_6$), and the active site $Cys^{105}\rightarrow$Ser mutation in the large subunit ($Cys^{105}\rightarrow$Ser-80k-$CHis_6$), have been described previously [17,18].

Rat 28 kDa Subunit, 28k and $NHis_{10}$-28k

In order to obtain the full-length rat calpain small subunit cDNA, a λgt11 rat aorta cDNA library was screened with cDNA for the rat Δ86 small subunit. The inserts from two positively-hybridizing plaque-purified λ clones were cloned into pUC19 by means of PCR with forward and reverse λ primers, followed by *Eco*RI digestion and ligation into the vector. One of these

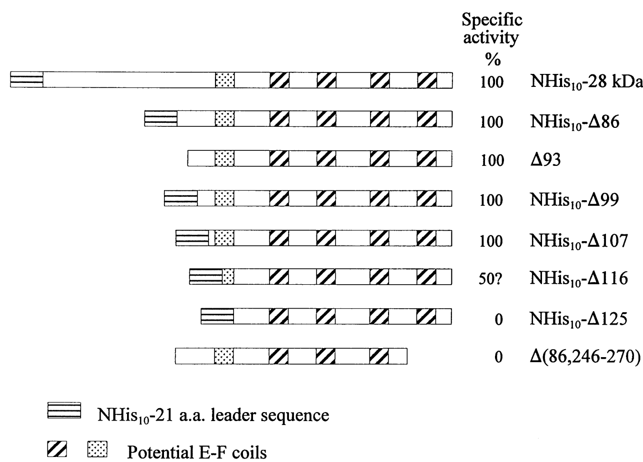

Figure 2 Diagram of the N- and C-terminal truncations of the small subunit of rat calpain

The diagonally hatched areas represent the Ca^{2+}-binding loops in the E-F hand motifs, as usually assigned [6], and the dotted areas represent a fifth loop suggested recently [8]. The N-terminal His-tag ($NHis_{10}$) is shown with horizontal hatching. The subunits are identified by the number of amino acids deleted from the full-length small subunit, ignoring the His-tag which adds a further 21 residues; they all have an unaltered C-terminus except for Δ(86,246–270), where 25 C-terminal residues have been removed. The subunit Δ93 has not been expressed in *E coli*, but it is included to indicate the assumed natural autolysis product of the small subunit. The specific activity of the corresponding heterodimer for each small subunit is given as a percentage of the control 80k-$CHis_6$/Δ86.

pUC19 clones contained 1.1 kb of sequence from nt 47 of the full-length coding sequence to a polyadenylation site, and was identical in the common region to the Δ86 cDNA described previously [16]. The other pUC19 clone clearly derived from a cloning artefact at some earlier stage, since it contained several fragments of small subunit coding sequence in a scrambled order. Fortunately however, the clone included the expected missing 5′ sequence. On the basis of this information, using a 2-fold degenerate sense primer at the initiation codon, gctgcacatatgttcttggtgaa(c/t)tcg (*Nde*I), and an antisense primer at the stop codon, tcaggtcaaagcttcacagtgagcagtgaggc (*Hin*dIII), the complete small subunit coding region was amplified from fresh rat lung total RNA by means of reverse transcription PCR (*Taq* Extender, Stratagene), yielding a single strong band on agarose gel electrophoresis. This was cloned by means of *Nde*I/*Hin*dIII digestion and ligation into pACpET-24 for expression as the unmodified subunit. It was also cloned into pET-16b(+), which provided sequence coding for a 21 amino acid $NHis_{10}$-leader peptide ($MGH_{10}SSGHIEGRH$) fused to the rat 28 kDa coding sequence, and then this construct was transferred by means of *Xba*I/*Hin*dIII digestion into pACpET-24. Figure 1 shows the coding and deduced amino-acid sequences of the complete rat calpain small subunit, together with the introduced *Nde*I and *Hin*dIII restriction sites. The sequence codes for a protein of 270 amino acids, with a predicted molecular mass of 28460 Da (here referred to as 28k). This sequence, except for the first 12 bp, has also been obtained independently by Sorimachi et al. [19]. The sequence obtained here agrees completely with that report, except for the *Eco*RI site early in the coding sequence [19], which was altered in our work by the degenerate base in the PCR primer.

Modifications of the rat calpain small subunit

Modifications of the rat calpain small subunit Δ86, Δ99, Δ107, Δ116, Δ125 and Δ(86,246–270), with and without an N-terminal His-tag, are shown in Figure 2.

The 21 kDa fragment, Δ86, containing domain VI of calpain, was originally constructed with an initiating methionine to give the N-terminal sequence $Met-His^{88}$, which is seven residues longer than the assumed autolysis product, Δ93 [16]. The natural autolysis product has not been characterized from rat, but is assumed to be Δ93 on the basis of sequence comparison with the autolysis products from bovine, pig and rabbit calpain small subunits [4,20,21]. The initiating methionine in Δ86 was retained on expression in *E. coli*, as demonstrated by amino acid sequencing [16]. Δ86 therefore has a predicted molecular mass of 21257 Da, which was confirmed by electrospray MS (observed value 21263 Da). The autolysis product Δ93 has been labelled traditionally as 18 kDa, but should be described more accurately as 20 kDa, since in porcine calpain, for example, it has a molecular mass of 20263 Da [9], and the predicted Δ93 in rat has a molecular mass of 20390 Da.

Site-directed mutagenesis was carried out with the cDNA for Δ86 in the vector pET-20b(+), since this vector contains the f1 origin of replication for synthesis of single-strand cDNA, and the products were checked by DNA sequencing. N-terminal truncations were made by introduction of a second *Nde*I site at the appropriate positions, followed by transfer of the shortened *Nde*I–*Hin*dIII fragment into pACpET-24. A $Glu^{100} \rightarrow Val$ mutation was also introduced into Δ99, both to maintain uniformity with the other truncated forms and because the alignment shows that valine is present in this position in the calpain small subunit of other species [19]. The resultant constructs for Δ99, Δ107 and Δ116 (Figure 2) all start with the sequence Met-Val, and Δ125 starts with the sequence Met-Leu. It is assumed that these initiating methionine residues are lost on expression in *E. coli*, as predicted for valine and leucine in the second position [22]. The C-terminal truncation [Met^{87}-Phe^{245}, Δ(86,246–270)] was produced similarly from Δ86 by introduction of a new stop codon and *Hin*dIII site in the desired positions. The mutagenic antisense primers, and their restriction sites (underlined) were: Δ99, 5′-P-tcctgaactgacggaccatatgactctcgttggcc-3′ (*Nde*I); Δ107, 5′-P-agccagctggaccatatgtttcctgaactg-3′ (*Nde*I); Δ116, 5′-P-ctgtagcgctgaccatatggtcgtctccagccag-3′ (*Nde*I); Δ125, 5′-P-gaccttgttgag catatgcatgagttctgt-3′ (*Nde*I); Δ(86,246–270), 5′-P-ccatttttgtc aagctttcagaaagcacggaac-3′ (*Hin*dIII; the stop codon is indicated with double underlining).

To facilitate purification of calpains with 1:1 subunit stoichiometry in cases where the level of expression of the small subunit was limiting, it was desirable to attach an N-terminal His-tag ($NHis_{10}$) to most of these small subunit constructs, as described above for the full-length rat calpain small subunit, and these are designated $NHis_{10}$-Δ86, etc.

Expression, purification and assay of calpain

Appropriate pairs of large subunit cDNA in pET-24d(+) and small subunit cDNA in pACpET-24 were co-expressed in *E. coli* BL21(DE3), and the resultant calpains were purified on columns of DEAE–Sepharose and Ni-nitrilotriacetic acid–agarose at 4 °C, and Q-Sepharose FPLC at room temperature [17,18]. All procedures with calpain were performed in the presence of 10 mM 2-ME and 5 mM EDTA on ice or at 4 °C, unless otherwise stated. Calpain activity was assayed in 10 mM 2-ME/50 mM Tris/HCl, pH 7.6, containing 2 mg of casein and either 5 mM EDTA or 5 mM $CaCl_2$, in a final volume of 0.4 ml. The reaction was started

by the addition of the enzyme sample and was terminated after incubation for 30 min at 25 °C by the addition of 0.3 ml of ice-cold 10 % (w/v) trichloroacetic acid. After standing in ice, the samples were centrifuged at 1000 ***g*** for 10 min, and the A_{280} of the supernatants was recorded. A unit of enzyme activity is defined as an increase of 0.1 absorbance unit over 30 min in the Ca^{2+}-containing samples after subtraction of the corresponding EDTA control values.

Electrophoresis and immunoblotting

Gel electrophoresis in SDS/Tris/Tricine gels and immunoblot detection on nitrocellulose membranes were carried out as described previously, using colour development with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate [16,17]. Two antibody preparations were used on the immunoblots: (1) an affinity-purified polyclonal antibody to the rat m-calpain large subunit as described previously [23], and (2) polyclonal antisera against bacterially expressed Δ86 was raised in two rabbits, and the antibodies were purified by affinity chromatography on Δ86 linked to CNBr–Sepharose.

RESULTS

Production of rat (80k/28k) calpain

Co-expression of 80k-$CHis_6$ with the unmodified 28k small subunit provided approximately 10 500 units of calpain activity from 4 litres of cell culture under the standard conditions. Expression of 80k-$CHis_6$/$NHis_{10}$-28k gave a yield of approximately 23 600 units, indicating that the $NHis_{10}$-full-length small subunit was expressed at a higher level with than without a His-tag. These yields should be compared with the 36 000 units which are routinely obtained from 80k-$CHis_6$/Δ86 expression (Table 1).

Immunoblotting of samples taken during purification of 28 kDa-containing calpains showed that the 28 kDa subunit, with or without the $NHis_{10}$-leader sequence, was very largely intact after cell harvesting and sonication, but was partially degraded during the first purification step. This could be minimized but not completely prevented by the use of higher concentrations of EDTA, PMSF and soybean trypsin inhibitor during the extraction. The same degradation occurred during purification of the inactive mutant Cys^{105} → Ser-80k-$CHis_6$/$NHis_{10}$-28k, showing that it was not caused by calpain itself. No sign of degradation was detected in expression of the shorter forms of the small subunit lacking domain V.

Small subunit autolysis

The mixture of 28 kDa-derived small subunit proteins was functional, since all of the intermediate forms were rapidly autolysed to a homogeneous 20 kDa small subunit on exposure of the 80 k/24–28 k calpains to Ca^{2+}, a characteristic of natural calpains [9,10,21] (Figure 3). Formation of a 20 kDa fragment, which corresponds to the expected Δ93 observed in autolysis of natural calpain, strongly suggests that the partial degradation of the recombinant 28 kDa small subunit was N-terminal.

Table 1 Initial and final yields and specific activities of calpain with various small subunits

The initial yield is given as the total enzyme activity in 4 litres of *E. coli* culture, measured after DEAE–Sephacel chromatography, the first purification step. The results of a single expression are shown but each expression was repeated with similar results. The final yield is given both as total enzyme activity recovered after three purification steps using column chromatography and as a percentage of the initial yield.

Large subunit	Small subunit[a]	Initial yield (units of activity)	Final yield (units of activity)	Final yield (% of initial activity)	Specific activity (units per mg of protein)
80k-$CHis_6$	28k	10 600	–	–	885[b]
80k-$CHis_6$	$NHis_{10}$-28k	23 600	14 000	59	1720
80k-$CHis_6$	Δ86	36 000	21 600	60	1860
80k-$CHis_6$	Δ99	30 700	17 800	58	1775
80k	$NHis_{10}$-Δ86	14 000	8 600	62	1920
80k	$NHis_{10}$-Δ99	8 520	4 050	48	1770
80k	$NHis_{10}$-Δ107	6 030	1 080	18	1720
80k	$NHis_{10}$-Δ116	2 770	510	18	1000[c]
80k	$NHis_{10}$-Δ125	0[d]			
80k	Δ(86,246-270)	0[e]			

[a] The structures of the modified small subunits are shown in Figure 2.
[b] Not fully purified.
[c] This value is approximate because of the small amount of enzyme and the instability of the product.
[d] Some heterodimer was detected in the eluate of the first column.
[e] No heterodimer was detected.

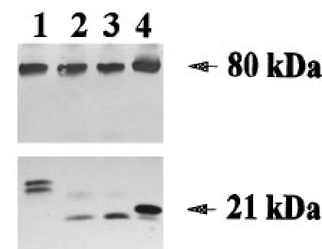

Figure 3 Autolysis of the 28 kDa small subunit to 20 kDa

A sample of 80k/$NHis_{10}$-28k (in fact containing heterogeneous small subunits) in 0.35 M NaCl/10 mM 2-ME/50 mM Tris/HCl, pH 7.6, was incubated at 20 °C with a net concentration of 2 mM $CaCl_2$ for 1 and 2 min, followed by addition of excess EDTA. Samples were run on Tris/Tricine gels and electroblotted on to nitrocellulose. The upper and lower halves of the blot were treated separately with antibodies to the two calpain subunits, followed by exposure to alkaline-phosphatase-conjugated second antibody and colour development. The lanes represent: 1, 80k/$NHis_{10}$-28k before autolysis; 2, 80k/$NHis_{10}$-28k after 1 min autolysis; 3, 80k/$NHis_{10}$-28k after 2 min autolysis; 4, standard 80k-$CHis_6$/Δ86. The arrows indicate the approximate molecular mass of the large (80 kDa) and small (Δ86, 21 kDa) subunits.

Effects of small subunit truncation

Table 1 shows the initial yields of activity for calpains with different locations of the His-tag and different sizes of small subunit, from 4 litres of *E. coli*. This initial yield is an approximate index of the relative level of expression of the two subunits. Table 1 shows, by comparison of expression of 80k-$CHis_6$/(Δ86 or Δ99) and 80k/($NHis_{10}$-Δ86 or -Δ99), that the large subunit

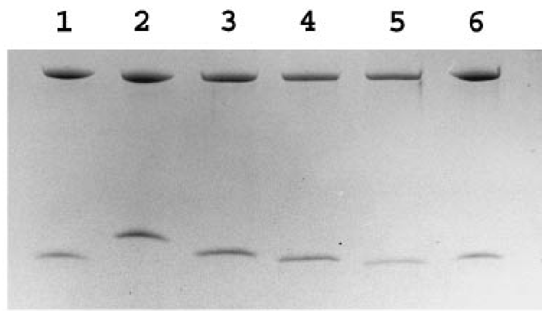

Figure 4 SDS/Tris/Tricine gel of calpains containing modified small subunits

The calpains were purified on columns of DEAE-Sepharose, Ni-NTA-agarose and Q-Sepharose (FPLC), and samples were run on a 12.5% (w/v) SDS/Tris/Tricine gel and stained with Coomassie Brilliant Blue R250. Lanes 1 and 6, 80k-CHis$_6$/Δ86; lane 2, 80k/NHis$_{10}$-Δ86; lane 3, 80k/NHis$_{10}$-Δ99; lane 4, 80k/NHis$_{10}$-Δ107; lane 5, 80k/NHis$_{10}$-Δ116.

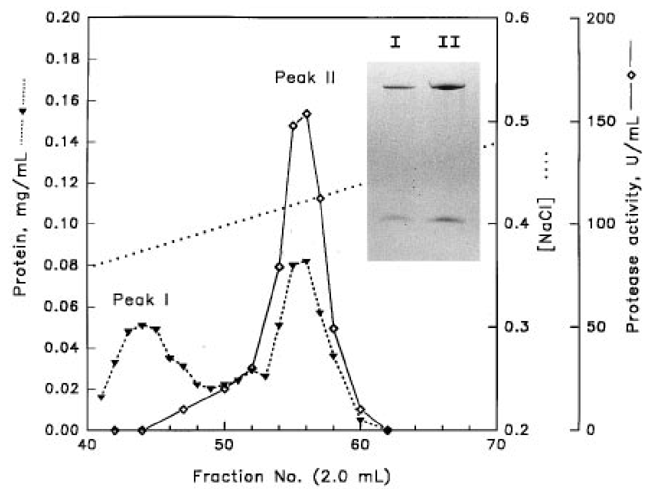

Figure 5 Formation of active and inactive heterodimers

The elution profile of the Q-Sepharose (FPLC) column, the final step in purification of 80k/NHis$_{10}$-Δ107, shows that this enzyme was eluted as two separate peaks, which appeared to be identical by gel electrophoresis; peak I had no activity, and peak II had a specific activity equal to that of standard 80k-CHis$_6$/Δ86. Protein (▼); specific activity (◇). Samples from these peaks, examined by gel electrophoresis and stained with Coomassie Brilliant Blue, are shown in the inset.

without a His-tag was expressed at a lower level than that with a His-tag. In contrast, the Δ86 form of the small subunit was expressed in a large molar excess with respect to either of these large subunits. Consequently, in co-expression with Δ86, expression of the large subunit was limiting in production of active calpain.

However, as the small subunit constructs became even smaller, with or without a His-tag, it was clear that their expression levels became lower than that of the large subunit and became limiting in the yield of active calpain, as indicated by the initial yields of activity following one purification step using column chromatography (Table 1). Very similar and progressively smaller amounts of activity were obtained from two series of constructs differing in location of the His-tag: (a) 80k-CHis$_6$/Δ99, Δ107, Δ116 and Δ125 (results not shown); and (b) 80k/NHis$_{10}$-Δ99, NHis$_{10}$-Δ107, NHis$_{10}$-Δ116 and NHis$_{10}$-Δ125. This showed that the NHis$_{10}$ group on the small subunit did not significantly alter subunit interactions.

The small subunit with a C-terminal truncation, Δ(86,246–270), was well expressed but did not form a heterodimer and did not generate activity.

In addition to initial yields, Table 1 shows the final yields and specific activities of the active calpains after three purification steps using column chromatography, at which point they were almost 100 % pure. Figure 4 shows a Coomassie-Blue-stained gel of the 80k/NHis$_{10}$ small subunit series together with a pure sample of 80k-CHis$_6$/Δ86. The latter has been shown, by N-terminal sequencing, to have a subunit ratio of 1 : 1. The results of the electrophoresis suggest that the subunit ratios of all these calpains were approximately 1 : 1. Although the activity expressed diminished with the length of the small subunit, the specific activities of 80k/NHis$_{10}$-Δ86, -Δ99 and -Δ107 were the same as that of 80k-CHis$_6$/Δ86; the specific activity of 80k/NHis$_{10}$-Δ116 was almost 50 % lower, but this value is equivocal because of the instability of this enzyme on the Q-Sepharose column in the final purification step. A small amount of the heterodimer 80k/NHis$_{10}$-Δ125 was present in the eluate of the DEAE–Sepharose column (immunoblot not shown), but no activity could be detected, and the heterodimer was not stable to subsequent purification on Ni-nitrilotriacetic acid and Q-Sepharose columns.

It appeared also that increasing the extent of N-terminal deletion tended to promote formation of an inactive heterodimer, while reducing formation of active heterodimer. Figure 5 shows the elution profile of 80k/NHis$_{10}$-Δ107 from the Q-Sepharose column in the final step of purification. Peak I contained no activity and peak II contained calpain with a specific activity of 1720 units per mg of protein, but the gel (and an immunoblot, which is not shown) showed that both peaks contained both subunits with identical stoichiometry (Figure 5, inset). Similar Q-Sepharose elution profiles were obtained for each of these calpains, but the ratio of protein in the inactive peak I to protein in the active peak II increased progressively from approximately 0.1 in 80k/NHis$_{10}$-Δ86 to 1.2 in 80k/NHis$_{10}$-Δ116.

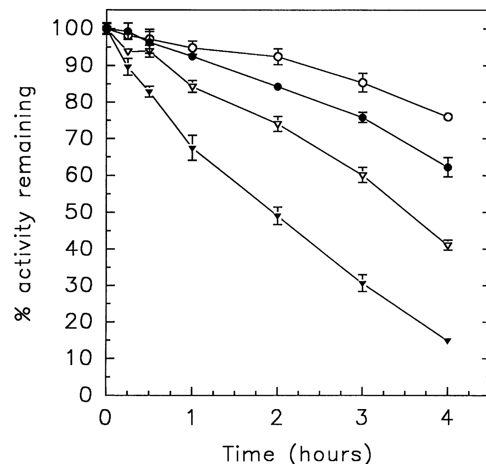

Figure 6 Relative stability of calpains with truncated small subunits

The calpains (0.05–0.1 μM) were incubated in 1 mM EDTA/0.4 M NaCl/10 mM 2-ME/50 mM Tris/HCl, pH 7.6, containing casein (5 mg/ml), at 37 °C for 0–4 h. The enzyme activity remaining was measured as described in the Experimental section at 25 °C in the presence of 5 mM Ca^{2+}. The results show the activity remaining (means ± S.D. of triplicate assays) and are expressed as a percentage of the original activity. 80k/NHis$_{10}$-Δ86, (○); 80k/NHis$_{10}$-Δ99, (●); 80k/NHis$_{10}$-Δ107, (▽); 80k/NHis$_{10}$-Δ116, (▼).

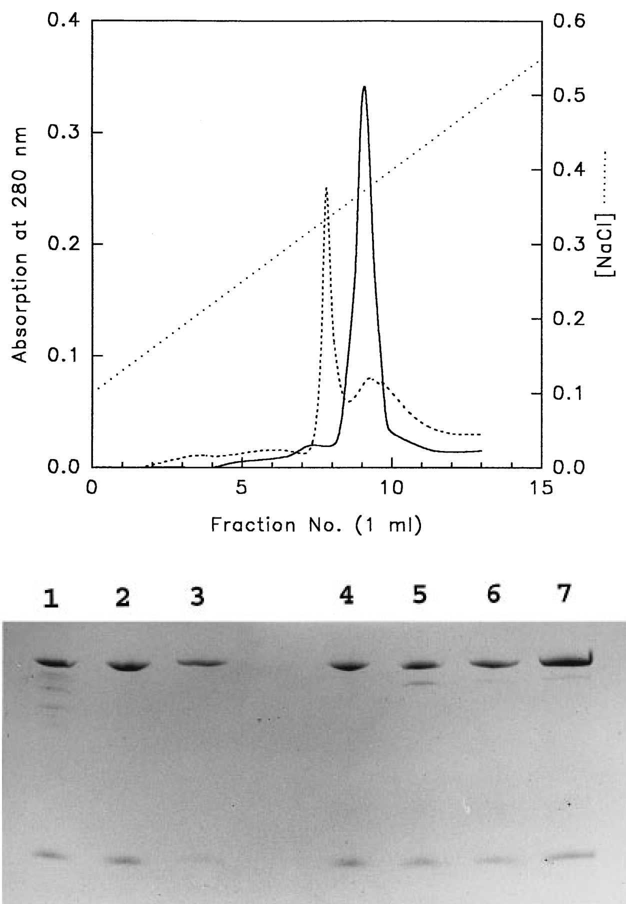

Figure 7 Ion-exchange chromatography of $Cys^{105} \rightarrow$ Ser-80k-$CHis_6/\Delta 86$ with or without Ca^{2+}

Upper panel; samples of $Cys^{105} \rightarrow$ Ser-80k-$CHis_6/\Delta 86$ calpain (0.6 mg) were applied to an HR 5/5 MonoQ FPLC column in 50 mM Tris/HCl, pH 7.6/0.1 M NaCl/10 mM 2-ME/0.1% (v/v) Triton-X 100, with the addition of either 2 mM EDTA (—) or 3 mM Ca^{2+} (– –), and the columns were eluted with a gradient of 0.1–1 M NaCl (· · · ·) in the same buffer, in a total volume of 30 ml. Fractions of 1 ml were collected. The lower panel shows a Coomassie-Blue-stained gel. Lanes 1–7 were loaded with approximately equal amounts of protein, lanes 1–3 contained samples of fractions 8–10 from the EDTA column, and lanes 4–7 contained samples of fractions 8–11 from the Ca^{2+} column.

Stability of the heterodimers

In addition to the decline in the initial yield, the percentage recovery of activity over the three-column purification protocol also became smaller as the size of the small subunit decreased (Table 1), suggesting that the activity was progressively less stable with the more N-terminally deleted small subunits. The differences in stability were further demonstrated by incubation in 0.4 M NaCl at 37 °C, where the rate of loss of activity increased as the size of the small subunit decreased (Figure 6).

Ca^{2+} titrations

The Ca^{2+}-requirements for hydrolysis of casein by many of these calpains (80k-$CHis_6$-$\Delta 86$, -$\Delta 99$, and -$\Delta 107$; 80k/$NHis_{10}$-28k, $NHis_{10}$-$\Delta 86$, $NHis_{10}$-$\Delta 99$) were measured under carefully controlled conditions and did not differ significantly. The value of $[Ca^{2+}]_{0.5}$ for all of these calpains was close to 350 μM (titration curves not shown). The details of the Ca^{2+}-titrations and the relationships between autolysis and Ca^{2+}-requirement have been described elsewhere [10a].

Maintenance of subunit association in the presence of Ca^{2+}

Samples of $Cys^{105} \rightarrow$ Ser-80k-$CHis_6/\Delta 86$ (0.6 mg) were applied to a MonoQ FPLC column and eluted, at room temperature, with an NaCl gradient (0–1.0 M) in 50 mM Tris/HCl, pH 7.6/0.1% (v/v) Triton X-100, and either 2 mM EDTA or 3 mM Ca^{2+}. Inactive $Cys^{105} \rightarrow$ Ser-calpain was used in order to exclude autolysis, and Triton X-100 was added to minimize Ca^{2+}-induced aggregation [13]. Active 80k-$CHis_6/\Delta 86$ was recovered in high yield under these conditions, when run in the presence of EDTA, and a single peak of $Cys^{105} \rightarrow$ Ser-80k-$CHis_6/\Delta 86$ was observed (Figure 7, upper panel). In the presence of Ca^{2+}, the principal peak of $Cys^{105} \rightarrow$ Ser-80k-$CHis_6/\Delta 86$ was eluted a little earlier in the salt gradient, consistent with a reduction in net negative charge caused by binding of some Ca^{2+}, and some tailing of heterodimer elution occurred, probably due to aggregation which was not wholly prevented by Triton X-100. The small subunit remained associated with the large subunit both in the presence and absence of Ca^{2+}, as indicated by the Coomassie Blue-stained gel (Figure 7, lower panel). When chromatographed alone on a MonoQ column, $\Delta 86$ was eluted in fractions 3–4 in the presence of EDTA, and was not retained in the presence of Ca^{2+}. It was concluded that the subunits in $Cys^{105} \rightarrow$ Ser-80k-$CHis_6/\Delta 86$ remained associated in the presence of Ca^{2+}.

DISCUSSION

Expression of calpain with a 28 kDa subunit

Most of our recent work has been done with the $\Delta 86$ form of the small subunit, which is highly expressed in *E. coli*. It was of interest, however, to complete the cloning of the small subunit which has, in the meantime, been reported independently [19], and to show that it could generate active calpain. Some activity was recovered following co-expression of the large subunit with full-length small subunits, but the lower amount of activity, in comparison with expression of 80k-$CHis_6/\Delta 86$, reflects the relatively poor expression of the full-length small subunit in comparison with the domain VI fragment, $\Delta 86$. These calpains have not yet been studied in great detail because of partial degradation of the complete small subunit in the early steps of purification. The resultant heterogeneous small subunits were however functional, since they were autolysed as expected to a homogeneous 20 kDa form (Figure 3). No degradation was observed during purification of calpains with $\Delta 86$ and shorter forms of the small subunit, suggesting that the glycine-rich domain V is particularly prone to attack by other proteases in *E. coli* lysates. The same is probably true for calpains isolated from natural sources, in which the small subunits were often apparently degraded or absent [1]. It was not possible to document this fully at the time for two reasons: the small subunits stain poorly in Coomassie Brilliant Blue, especially when they are present as mixtures of several degradation products; and specific high-affinity antibodies to the small subunit were not available.

Expression of calpain with truncated small subunits

It was already clear that residues 1–93 of the small subunit, which are removed during autolysis, are not required either for calpain activity or for formation of active calpain in *E. coli*,

although they must have some role in regulating calpain activity in eukaryotic cells. It was therefore of interest to study the effects of further truncations of the small subunit.

The yields of activity of recombinant calpains reflect two factors: the relative levels of expression of the two subunits, which vary widely and unpredictably, and their ability to form functional heterodimers. The high level of expression of Δ86 clearly promotes formation of active heterodimeric calpain, and excess Δ86 is easily separated from recombinant m-calpain by column chromatography. As the small subunit constructs became smaller, however, their level of expression became limiting, as indicated by immunoblots of the crude lysates (results not shown), and by the initial yields of active enzyme. Therefore $NHis_{10}$ was placed on the small subunit constructs to permit purification of these calpains with 1:1 stoichiometry. Comparison of results obtained with and without $NHis_{10}$ on the small subunit showed that this tag had no significant effect on calpain-specific activity or on subunit binding.

Progressive truncation of the small subunit has shown that virtually all of the natural autolysis product (Δ93) of the small subunit is important for formation of stable active heterodimer in *E. coli*. The specific activity of calpain was not significantly altered as the small subunit deletions proceeded up to Δ107, or even up to Δ116, so that the residues between 87 and 116 are clearly not directly involved in calpain catalysis. These residues do however contribute in some way to stability and correct conformation of the heterodimers. This was shown by the loss of activity during purification (Table 1), which became more marked as the small subunit diminished in size, and also by the declining stability of the heterodimers at 37 °C in 0.4 M NaCl (Figure 6). Consistent with this trend, the final N-terminal truncation, $NHis_{10}$-Δ125, was able to form only an inactive and unstable heterodimer.

In addition, inactive heterodimers were formed in greater proportions with small subunits having longer N-terminal deletions. The differences between the active and inactive heterodimers, the reasons for their separation on Q-Sepharose and the nature of the instability of the active calpains are not yet clear but all of these observations indicate that the N-terminal regions of Δ86 are important in generating active calpain. One factor in the instability *in vitro* may be high ionic strength. There are ten charged residues between positions 94 and 116, which could form salt links, including some possibly to the large subunit. The binding in this region would be weakened both by progressive removal of these residues, and also by the presence of high salt concentrations which mask charge interactions. It is not yet known what contacts these residues make with other parts of the molecule, but since autolysis of the small subunit occurs at Glu^{93}-Ala^{94}, small subunit residues between 94 and 116 are likely to be close to the active site in domain II.

The N-terminal truncations do not directly involve the four E-F hands (residues 154–165, 184–195, 219–230 and 249–260) (Figures 1 and 2) usually described in domain VI, but do encroach on a fifth more N-terminal E-F hand (residues 111–122), which was proposed recently on the basis of an alternative alignment [8], and demonstrated by X-ray crystallography [24].

C-terminal truncation of Δ86 showed that residues between 246 and 270 are essential for subunit binding, as previously shown by reconstitution *in vitro* [6]. While the lack of activity of Δ(86,246–270) could be due to incorrect folding, this construct was soluble and very well expressed, and is therefore likely to be properly folded. The failure to support calpain activity is consistent with the X-ray crystallographic evidence which suggests that the deleted C-terminal residues represent a major subunit binding site [24].

Subunit dissociation in the presence of Ca^{2+}

These results have shown that at least some portion of the calpain small subunit is essential for formation of active heterodimer during expression in *E. coli*. It has, however, been suggested that the function of the small subunit is solely to assist folding of the large subunit, and in support of this idea it was reported that the calpain large subunit dissociated reversibly from the small subunit on exposure to Ca^{2+} and was active as a monomer [12,13]. Definitive experiments on this point are made very difficult by Ca^{2+}-induced autolysis and aggregation. The observation (Figure 7) that inactive $Cys^{105} \rightarrow Ser$-$80kCHis_6/\Delta86$ (which cannot autolyse) did not dissociate in the presence of Ca^{2+} does not appear to support the suggestion of Ca^{2+}-induced subunit dissociation. In agreement with our findings, it has also been reported that subunit dissociation could not be detected in experiments involving immunoprecipitation of calpains with subunit-specific monoclonal antibodies [25].

In summary, the results described here have shown that small subunit residues between 94 and 116, and between 246 and 270, are important for subunit binding. It is hoped that this information, together with the X-ray crystallography of the homodimer of Δ86 [26], will contribute to an understanding of subunit interactions in calpain.

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REFERENCES

1 Croall, D. E. and DeMartino, G. N. (1991) Physiol. Rev. **71**, 813–847
2 Goll, D. E., Thompson, V. F., Taylor, R. G. and Zalewska, T. (1992) BioEssays **14**, 549–556
3 Ohno, S., Emori, Y., Imajoh, S., Kawasaki, H., Kisaragi, M. and Suzuki, K. (1984) Nature (London) **312**, 566–570
4 Emori, Y., Kawasaki, H., Imajoh, S., Kawashima, S. and Suzuki, K. (1986) J. Biol. Chem. **261**, 9472–9476
5 Minami, Y., Emori, Y., Kawasaki, H. and Suzuki, K. (1987) J. Biochem. (Tokyo) **101**, 889–895
6 Minami, Y., Emori, Y., Imajoh-Ohmi, S., Kawasaki, H. and Suzuki, K. (1988) J. Biochem. (Tokyo) **104**, 927–933
7 Zimmerman, U.-J. F. and Schlaepfer, W. W. (1988) Arch. Biochem. Biophys. **266**, 462–469
8 Theopold, U., Pinter, M., Daffre, S., Tryselius, Y., Friedrich, P., Nassel, D. R. and Hultmark, D. (1995) Mol. Cell. Biol. **15**, 824–834
9 Crawford, C., Brown, N. R. and Willis, A. C. (1993) Biochem. J. **296**, 135–142
10 Saido, T. C., Nagao, S., Shiramine, M., Tsukaguchi, M., Yoshizawa, T., Sorimachi, H., Ito, H., Tsuchiya, T., Kawashima, S. and Suzuki, K. (1994) FEBS Lett. **346**, 263–267
10a Elce, J. S., Hegadorn, C. and Arthur, J. S. C. (1997) J. Biol. Chem. **272**, 11268–11275
11 Kikuchi, T., Yumoto, N., Sasaki, T. and Murachi, T. (1984) Arch. Biochem. Biophys. **234**, 639–645
12 Yoshizawa, T., Sorimachi, H., Tomioka, S., Ishiura, S. and Suzuki, K. (1995) FEBS Lett. **358**, 101–103
13 Yoshizawa, T., Sorimachi, H., Tomioka, S., Ishiura, S. and Suzuki, K. (1995) Biochem. Biophys. Res. Comm. **208**, 376–383
14 Nishimura, T. and Goll, D. E. (1991) J. Biol. Chem. **266**, 11842–11850
15 Imajoh, S., Kawasaki, H. and Suzuki, K. (1987) J. Biochem. (Tokyo) **101**, 447–452
16 Graham-Siegenthaler, K., Gauthier, S., Davies, P. L. and Elce, J. S. (1994) J. Biol. Chem. **269**, 30457–30460
17 Elce, J. S., Hegadorn, C., Gauthier, S., Vince, J. W. and Davies, P. L. (1995) Protein Eng. **8**, 843–848
18 Arthur, J. S. C., Gauthier, S. and Elce, J. S. (1995) FEBS Lett. **368**, 397–400
19 Sorimachi, H., Amano, S., Ishiura, S. and Suzuki, K. (1996) Biochim. Biophys. Acta **1309**, 37–41
20 McClelland, P., Lash, J. A. and Hathaway, D. R. (1989) J. Biol. Chem. **264**, 17428–17431

21 Imajoh, S., Kawasaki, H. and Suzuki, K. (1986) J. Biochem. (Tokyo) **100**, 633–642
22 Kendall, R. L., Yamada, R. and Bradshaw, R. A. (1990) Methods Enzymol. **185**, 398–407
23 DeLuca, C. I., Davies, P. L., Samis, J. A. and Elce, J. S. (1993) Biochim. Biophys. Acta **1216**, 81–93
24 Blanchard, H., Grochulski, P., Li, Y., Arthur, J. S. C., Davies, P. L., Elce, J. S. and Cygler, M. (1997) Nature (London) Struct. Biol. **4**, 532–538
25 Zhang, W. and Mellgren, R. L. (1996) Biochem. Biophys. Res. Comm. **227**, 890–896
26 Blanchard, H., Li, Y., Cygler, M., Kay, C. M., Arthur, J. S. C., Davies, P. L. and Elce, J. S. (1996) Protein Sci. **5**, 535–537

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