Studies of the active site of m-calpain and the interaction with calpastatin

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Calpain autolyses in the presence of Ca^{2+}. In the case of m-calpain (80 + 30 kDa) the first product is an 80 + 18 kDa species which has an intact large subunit and the C-terminal Ca^{2+}-binding domain of the small subunit. It was possible to bind E64 into the active site of calpain in the presence of Ca^{2+} before cleavage of either calpain subunit. This suggests that the active site is functional before any autolysis has occurred and that calpain is not a proenzyme. Prolonged autolysis generates several fragments including a 42 kDa active-site domain fragment that showed no proteolytic activity and Ca^{2+}-binding domain fragments. Some of the Ca^{2+}-binding domain fragments were found to exist as heterodimers (23 + 18 kDa and 22 + 18 kDa), with the Ca^{2+}-binding domain of the large subunit interacting with the Ca^{2+}-binding domain of the small subunit. These species were true heterodimers, as they showed co-elution of the two Ca^{2+}-binding domains on ion-exchange and gel-filtration chromatography, and immunoprecipitation of both polypeptides with an antiserum specific for the small-subunit Ca^{2+}-binding domain. The generation of the dimer species after only 15 min autolysis suggests that the interaction between the Ca^{2+}-binding domains is present in the native calpain structure. The interaction of calpain with calpastatin was investigated using an assay based on binding to calpastatin–Sepharose and a competitive binding assay. Calpain, active-site-blocked calpain and calpain fragments generated by autolysis were studied. Calpain bound to calpastatin in the presence of Ca^{2+}; however, the isolated active-site-containing 80 kDa large subunit (proteolytically inactive), a 42 kDa active-site-containing fragment (proteolytically inactive) and Ca^{2+}-binding domain fragments of calpain did not. Active-site-blocked calpain bound to calpastatin, but with an affinity reduced by approximately two orders of magnitude when compared with native calpain.

INTRODUCTION

Calpains are intracellular Ca^{2+}-activated cysteine proteinases. They are thought to have a role in modulating signal transduction and in cytoskeletal modifications following cell activation (Croall and DeMartino, 1990). Two forms of the enzyme are well characterized: μ-calpain (calpain I) which is active at micromolar Ca^{2+} concentration *in vitro* and m-calpain (calpain II) which requires millimolar Ca^{2+} *in vitro*. The enzymes have two subunits: one of 80 kDa and one of 30 kDa. The 30 kDa subunits of μ-calpain and m-calpain are identical, but the 80 kDa subunits have different amino acid sequences in the two forms of the enzyme. The 80 kDa subunit has four domains including a papain-like active-site domain and a C-terminal calmodulin-like Ca^{2+}-binding domain. The 30 kDa subunit has a glycine-rich N-terminal domain which is involved in interaction with phospholipid and a C-terminal calmodulin-like Ca^{2+}-binding domain.

Calpains are only active in the presence of Ca^{2+}. On binding Ca^{2+}, calpains are activated and begin to autolyse. Autolysis results initially in an increase in Ca^{2+} sensitivity of the enzyme (Suzuki et al., 1981) and ultimately in the loss of enzyme activity (Crawford et al., 1987). For m-calpain, cleavage of the small subunit occurs after less than 1 min incubation with Ca^{2+} and generates an 18 kDa C-terminal fragment. Over longer periods of incubation with Ca^{2+}, cleavages occur in the large subunit of calpain generating fragments of 30–55 kDa (Crawford et al., 1987). On the basis of kinetic studies, it has been suggested that calpain is a proenzyme and only active after autolysis of the small subunit (Coolican et al., 1986; DeMartino et al., 1986; Inomata et al., 1986). Conversely, Cong et al. (1989) propose that unautolysed m-calpain is active.

Calpastatin is a specific inhibitor of calpain. The primary sequence of pig heart calpastatin shows it has 713 amino acid residues, which can be divided into five domains of approx. 140 amino acid residues (Takano et al., 1988). A unique N-terminal domain is followed by four homologous domains. Each of the four homologous domains show inhibitory activity against calpain (Maki et al., 1987). Ca^{2+} is required for the inhibition of calpain by calpastatin (Murachi et al., 1981). Studies of the individual inhibitory domains of calpastatin show that the interaction with calpain is competitive with nanomolar K_i (Maki et al., 1988). This suggests that calpastatin binds to the active site of calpain. However, calpain in which the active-site cysteine has been modified by oxidation or reaction with iodoacetic acid will bind to calpastatin–Sepharose columns (Cottin et al., 1983; Kapprell and Goll, 1989). This suggests that interaction of calpastatin with calpain does not depend exclusively on the area in the immediate vicinity of the active-site cysteine.

The present paper describes a study of m-calpain autolysis and investigation of the interaction of calpain, calpain subunits, calpain autolytic fragments and active-site-blocked calpain with calpastatin. The aim was to learn more about the functioning of the active site of m-calpain and the structures in calpain that are necessary for binding to calpastatin.

EXPERIMENTAL

Materials

E64 [*trans*-epoxysuccinyl-leucylamido-(4-guanidino)butane], phenylmethanesulphonyl fluoride (PMSF), soyabean trypsin inhibitor, leupeptin, pepstatin A, aprotinin and iodoacetic acid were from Sigma, Poole, Dorset, U.K. Radioiodinated Bolton and Hunter reagent was from Amersham International plc, Amersham, Bucks., U.K. Protein A–Sepharose was from Bioprocessing Ltd., Consett, Durham, U.K. DEAE-Sepharose,

Abbreviations used: E64, *trans*-epoxysuccinyl-leucylamido-(4-guanidino)butane; PMSF, phenylmethanesulphonyl fluoride.

Blue–Sepharose and CNBr-activated Sepharose were from Pharmacia Ltd., Milton Keynes, Beds., U.K.

Preparation of pig kidney m-calpain

m-Calpain was purified from pig kidney as described by Crawford et al. (1990). Enzyme activity was determined using azocasein as a substrate by the method of Crawford et al. (1987). In this assay, activity is assessed by measuring absorbance at 360 nm due to acid-soluble azocasein peptides generated after 30 min incubation with calpain. Complete digestion of 1 μmol of azocasein gives 16.4 absorbance units (S.E.M. = 0.8, $n = 3$) in the standard assay. The $k_{cat.}$ and K_m were determined to be 0.21 s^{-1} and 234 μM respectively. These values were found from an Eadie–Hofstee plot of data from two experiments; the plot had a correlation coefficient of 0.97.

Isolation of the calpain subunits

Calpain in 50 mM Tris/HCl, pH 7.5, containing 100 mM NaCl, 5 mM EDTA, 0.01 % monothioglycerol and 0.01 % NaN_3, was made 1 M in NaSCN and run on an h.p.l.c. gel-filtration column (Zorbax GF-250 column, 9.4 mm x 250 mm, 0.7 ml/min) equilibrated in 50 mM Tris/HCl, pH 7.5, containing 1 M NaSCN, 5 mM EDTA, 0.01 % monothioglycerol and 0.01 % NaN_3. Peaks (detected by absorbance at 280 nm) corresponding to the two separated subunits were collected and the samples dialysed sequentially against (1) 50 mM Tris/HCl, pH 7.5, containing 0.5 M NaSCN, 10 % glycerol, 5 mM EDTA, 0.01 % monothioglycerol and 0.01 % NaN_3, (2) buffer without NaSCN and (3) buffer without glycerol. The isolated 80 kDa subunit showed no detectable caseinolytic activity. This is in contrast with the observations of Imajoh et al. (1986) who found that the isolated subunit had residual activity.

Generation of active-site-blocked calpain used in the calpastatin–Sepharose-binding experiments

Calpain (approx. 4 nmol) was incubated with E64 (100-fold molar excess) or iodoacetic acid (1000-fold molar excess) in 300 μl of 50 mM Tris/HCl, pH 7.5, containing 100 mM NaCl, 0.01 % NaN_3, 5 mM free Ca^{2+} and 0.01 % monothioglycerol for 1 h at room temperature, followed by dilution to 500 μl and dialysis against 2 x 1 litre of 50 mM Tris/HCl, pH 7.5, containing 100 mM NaCl, 0.01 % NaN_3, 5 mM EDTA and 0.01 % monothioglycerol. The protein after blocking showed no detectable caseinolytic activity.

Production of antisera

A rabbit antiserum against pig kidney m-calpain was raised. Calpain (26 μg) in PBS with Freund's complete adjuvant (2 ml total volume) was injected subcutaneously at multiple sites into adult rabbits. Booster injections prepared in Freund's incomplete adjuvant were given at 1 and 6 months. Blood was taken 10 days after each booster injection. A rabbit antiserum against the 18 kDa C-terminal autolytic fragment of the small subunit of pig kidney m-calpain was also raised as follows. The 18 kDa fragment was prepared by autolysing calpain for 30 s in the presence of 5 mM Ca^{2+} and separating the 80 kDa and 18 kDa subunits by h.p.l.c. as described above for the intact calpain subunits. The 18 kDa fragment was cross-linked to thyroglobulin by reacting *m*-maleimidobenzoylsulphosuccinimide ester-treated thyroglobulin with $NaBH_4$-reduced protein as described by Rothbard et al. (1984). The 18 kDa fragment–thyroglobulin conjugate (200 μg per injection) was used to raise an antiserum as described for the anti-(m-calpain) serum.

Purification of calpastatin

Calpastatin was purified from pig heart using the rapid procedure described by Mellgren et al. (1988) with some modifications. Pig heart (approx. 1 kg of fresh tissue) was minced and homogenized in 2 litres of 50 mM Tris/HCl, pH 7.4, containing 5 mM EDTA, 0.01 % monothioglycerol, 10 μg/ml soyabean trypsin inhibitor, 0.7 μg/ml pepstatin A, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin and 0.1 mM PMSF (buffer X). The homogenate was then treated as described by Mellgren et al. (1988) except that Blue–Sepharose was used in place of Affi-gel Blue. As a final stage of purification, the active fractions from the Blue–Sepharose were concentrated by ultrafiltration to about 3 ml, diluted 10-fold with buffer X, and then run on a Mono Q HR5/5 column (Pharmacia Ltd.) equilibrated in buffer X and eluted with a 50 ml gradient from 0.05–0.30 M NaCl. The active fractions were pooled and stored frozen in the presence of 0.01 % NaN_3.

Calpastatin activity was measured by its ability to inhibit the activity of calpain assayed using azocasein. Calpastatins from rabbit skeletal muscle and from human liver are known to have low absorption coefficients (Imajoh et al., 1984; Nakamura et al., 1984). Therefore, in order to use A_{280} as a method for determining the protein concentrations of calpastatin solutions, the absorption coefficient for pig heart calpastatin was determined based on amino acid analysis. The samples for analysis were hydrolysed in gaseous constant-boiling HCl at 150 °C for 75 min. The amino acid composition was determined after precolumn formation of derivatives with phenyl isothiocyanate with the Waters Pico-Tag system. The protein concentrations of calpastatin solutions were thus found using absorbance at 280 nm and $A_{280} = 0.22$ for a 1 mg/ml solution in a 1 cm light path.

Binding assay using calpastatin–Sepharose

Calpastatin was coupled to CNBr-activated Sepharose as described by the manufacturers except that 1 mM PMSF was included in all buffers. The final preparation was suspended in 50 mM Tris/HCl, pH 7.5, containing 100 mM NaCl, 5 mM EDTA, 0.01 % monothioglycerol, 0.01 % NaN_3 and 1 mM PMSF. Control Sepharose was generated using the same procedure without the addition of calpastatin. The calpastatin–Sepharose was stored at 4 °C and used promptly. For the binding assay, the test protein (approx. 10 μg) was incubated with 40 μl of packed calpastatin–Sepharose in 110 μl of 50 mM Tris/HCl, pH 7.5, containing 100 mM NaCl, 0.01 % monothioglycerol, 0.1 % Tween 20, 2 mM PMSF and 5 mM free Ca^{2+} for 1 h at 4 °C. The supernatant was then removed, the Sepharose washed three times with 1 ml of the incubation buffer, the bound protein released with SDS sample buffer and analysed by SDS/PAGE.

Electrophoresis and Western blotting

SDS/PAGE was with gels of 12.5 % or 10 % (w/v) polyacrylamide and the buffer system described by Laemmli (1970). The samples were prepared by adding an equal volume of 125 mM Tris/HCl, pH 6.8, containing 20 % (v/v) glycerol, 10 % (w/v) SDS, 5 % (v/v) 2-mercaptoethanol and 5 % (v/v) saturated Bromophenol Blue, and incubating in a boiling-water bath for

5 min. The gels were stained with Coomassie Brillant Blue, dried down for autoradiography or used for Western blotting. In the last case, gels were blotted on to nitrocellulose soaked in 25 mM Tris/192 mM glycine buffer containing 10% (v/v) methanol using a LKB 2117 Multiphore II semi-dry blotting apparatus (180 mA for 1 h). Protein bands were visualized using a biotinylated antibody/streptavidin biotinylated alkaline phosphatase detection kit (Amersham International).

Amino acid sequence analysis

Amino acid sequence analysis was performed as described previously (Crawford et al., 1990). Briefly, the samples were subjected to SDS/PAGE, blotted on to poly(vinylidene difluoride) membranes and the bands of interest excised after staining with Ponceau S. Sequence analysis was with an Applied Biosystems 470A/120A protein sequencer with on-line phenylthiohydantoin analysis.

Mass spectroscopy

Electrospray mass spectra were measured on a VG BIO Q triple quadruple atmospheric-pressure mass spectrometer equipped with an electrospray interface (VG Biotech, Tudor Road, Altrincham, Cheshire, U.K.). Samples (10 μl) were injected into the electrospray source via a loop injector (Rheodyne 5717) as a solution (12.5 pmol/μl) in aq. 10 mM Tris/HCl, pH 7.5, containing 1 mM dithiothreitol/acetonitrile (1:1) and 1% formic acid at a flow rate of 2 μl/min (Applied Biosystems model 140A dual-syringe pump). The mass spectrometer was scanned over the mass range 650–1550 Da. The instrument was calibrated with myoglobin (20 pmol/μl; molecular mass 16951.5 Da).

RESULTS

Incubation of calpain in Ca^{2+} for 30 s converts the 80+30 kDa calpain into a 80+18 kDa form (Figure 1). The question of whether m-calpain is an active enzyme before autolysis was addressed by allowing the enzyme to react with E64 (a mechanism-based active-site-directed inhibitor of cysteine proteinases) before any autolytic cleavage. E64 (3.4 μmol) was added to calpain (14 nmol) in buffer A (50 mM Tris/HCl, pH 7.5, containing 100 mM NaCl, 5 mM EDTA, 0.01% monothioglycerol and 0.1% NaN_3). The solution was made 5 mM in free Ca^{2+} (final volume 2.3 ml), incubated for 1 h at room temperature and dialysed against 20 mM bis-Tris propane/HCl, pH 7.0, containing 200 mM NaCl, 5 mM EDTA, 0.01% monothioglycerol and 0.01% NaN_3 (buffer B). The sample was loaded on to a Mono Q HR5/5 f.p.l.c. column equilibrated in buffer B and the protein eluted with a 30 ml gradient from 0.26 to 0.38 M NaCl. During the reaction with E64, inhibition and autolysis both occur. The Mono Q column separates 80 kDa+30 kDa calpain from autolysed molecules. The 80 kDa+30 kDa calpain isolated from the Mono Q was inactive when tested with azocasein (it had less than 0.2% of the activity of native calpain). Thus this material is assumed to be active-site-blocked with E64. Previous work with a radioactive epoxide inhibitor demonstrated incorporation of radioactivity into m-calpain under similar conditions (Parkes et al., 1985). The active-site-blocked calpain appears to be identical with native calpain on SDS/PAGE (Figure 1). Amino acid sequence analysis was carried out on the subunits of calpain, 80+18 kDa autolysed calpain and E64-blocked calpain. The 80 kDa subunit of the autolysed calpain showed no sequence from 51 μg of material. The 80 kDa subunit of the autolysed m-calpain thus has a blocked N-terminus, as has the unautolysed protein. Similar observations were made on rabbit m-calpain (Imajoh et al.,

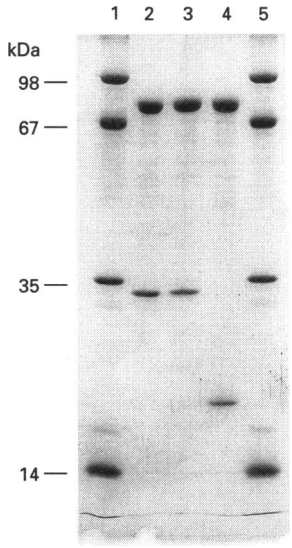

Figure 1 E64-blocked calpain and short (30 s) autolysis of calpain

Calpain (6.7 μg) in 50 mM Tris/HCl, pH 7.5, containing 100 mM NaCl, 5 mM EDTA, 0.01% monothioglycerol and 0.1% NaN_3 (buffer A) was made 5 mM in free Ca^{2+} and incubated for 30 s at room temperature (10 μl final volume). The reaction was stopped by adding 20 μl of SDS gel sample buffer and the sample analysed by SDS/PAGE (12.5% gel). Lanes 1 and 5, molecular-mass markers; lane 2, calpain; lane 3, E64-blocked calpain; lane 4, calpain autolysed for 30 s.

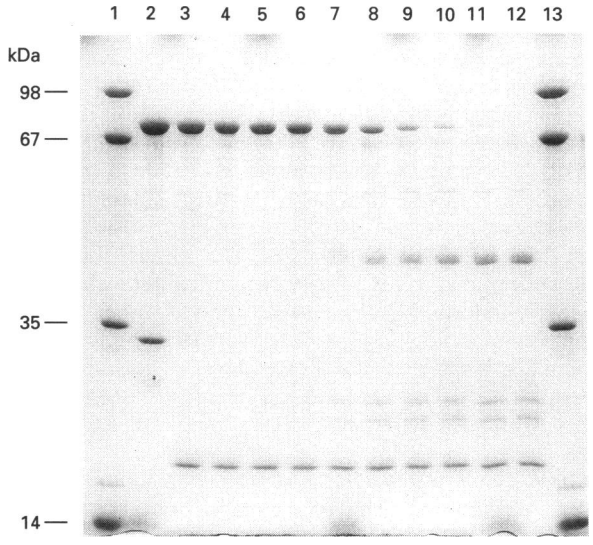

Figure 2 Autolysis time course

Calpain (60 μg) was incubated in 50 mM Tris/HCl, pH 7.5, containing 100 mM NaCl, 5 mM EDTA, 0.01% monothioglycerol, 0.01% NaN_3 with 5 mM free Ca^{2+} at room temperature. At various times samples (2 μg) were removed and analysed by SDS/PAGE (12.5% gels). Lanes 1 and 13, molecular-mass markers; lanes 2–12, samples taken at 0, 5, 15 and 30 s, 1, 5, 15 and 30 min, 1, 2 and 3 h.

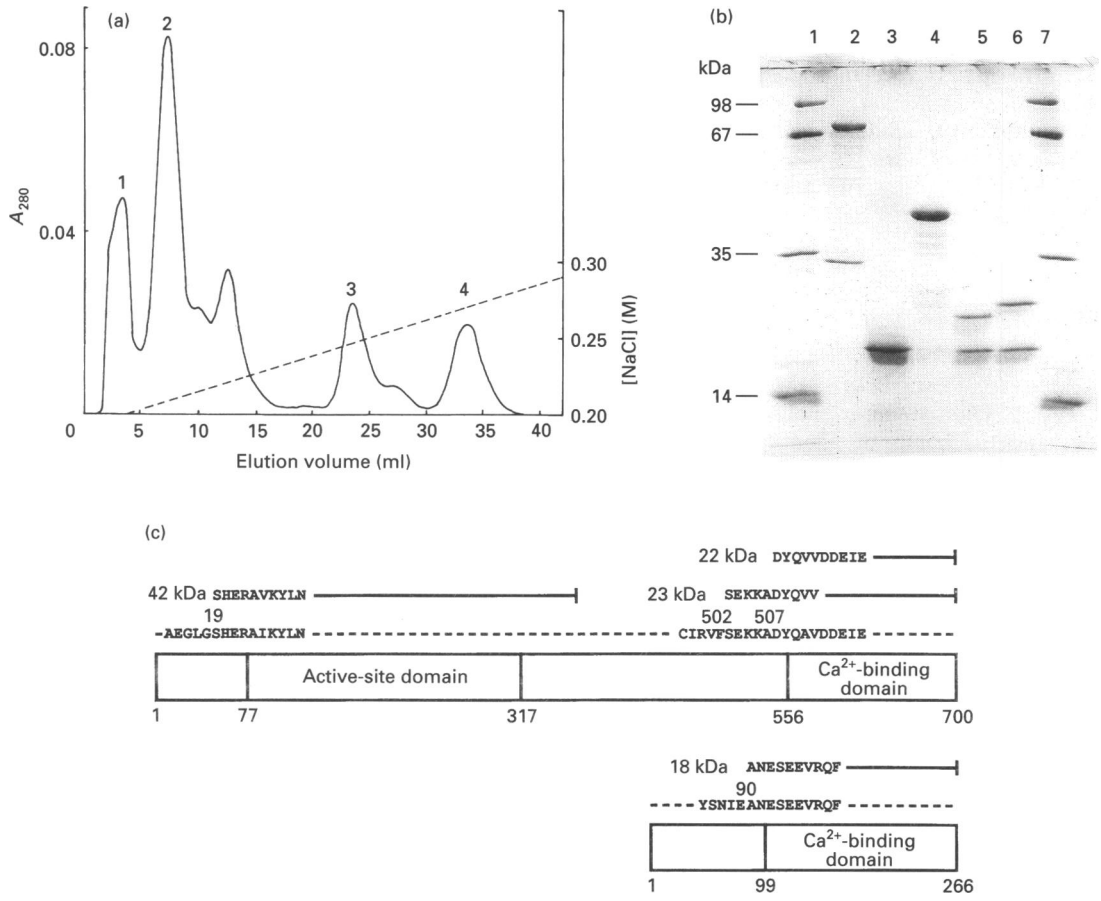

Figure 3 (a) Separation on Mono Q, (b) SDS/PAGE and (c) amino acid sequence analysis of calpain fragments generated after 2 h of autolysis

(**a**) Pools 1–4 were made corresponding to the peaks indicated. ——, A_{280}; – – – –, [NaCl]. (**b**) Lanes 1 and 7, molecular-mass markers; lane 2, calpain; lane 3, pool 1 (18 kDa); lane 4, pool 2 (42 kDa); lane 5, pool 4 (22+18 kDa); lane 6, pool 3 (23+18 kDa). (**c**) The sequences found are the upper sequences. The lowest sequence is the sequence derived from cDNA of human m-calpain for the large subunit and pig calpain for the small subunit.

1986). Sequence analysis showed that the 18 kDa fragment of autolysed calpain corresponds to residue 90 onwards of the small subunit (Crawford et al., 1990). These results demonstrate that the first autolytic event is cleavage of the m-calpain small subunit, and that this is complete before any degradation of the large subunit occurs. Sequence analysis of the large subunit of E64-blocked calpain showed no sequence from 60 μg of material; it therefore has a blocked N-terminus. The small subunit was similarly undegraded. (N.B. Where N-terminally blocked polypeptides were indicated from sequence analysis, other polypeptides from the same blot were sequenced with the expected results, confirming that blockage was not caused by the electrophoresis/blotting procedure.) These experiments demonstrate that calpain can react with the active-site-directed inhibitor E64 before autolysis.

Prolonged autolysis generates a number of calpain fragments as shown in Figure 2. The following sections describe the characterization of these fragments. This characterization was undertaken as autolysis generates fragments useful for examining the functions of various parts of the calpain molecule. To prepare completely autolysed enzyme, calpain (2 mg) was incubated in buffer A with 5 mM free Ca^{2+} for 2 h at room temperature and dialysed against buffer B. After 2 h the sample contains four major polypeptides of 42, 23, 22 and 18 kDa. These can be separated on a Mono Q HR5/5 column equilibrated in buffer B using a gradient from 0.20 to 0.29 M NaCl. The column profile and analysis of the peaks by SDS/PAGE are shown in Figures 3(a) and 3(b) respectively. The N-terminal amino acid sequence of each of the bands was determined and is shown in Figure 3(c). The position of the fragments in the molecule was deduced from the human m-calpain large-subunit sequence (Imajoh et al., 1988) and the pig small-subunit sequence (Sakihama et al., 1985). The calpain-cleavage sites show diverse primary sequences. The 42 kDa fragment begins at residue 19 of the large subunit, and is assumed from its molecular mass to extend into the third domain of the protein. It thus represents a fragment containing the active-site domain; however, the 42 kDa fragment showed no proteolytic activity (less than 0.2% that of native calpain). The 23 kDa and 22 kDa fragments include the C-terminal Ca^{2+}-binding domain of the large subunit and begin at residue 502 and 507 respectively. The 18 kDa fragments which were eluted in peaks 1, 3 and 4 on the Mono Q column (Figures 3a and 3b) had the same sequence, corresponding to the C-terminal Ca^{2+}-binding domain of the small subunit beginning at residue 90. This pattern of elution of the 18 kDa fragment from the Mono Q suggested that peak 1 corresponded to the free 18 kDa, and that peaks 3 and 4 were 18 kDa associated respectively with the 23 kDa and 22 kDa large-subunit fragments.

In order to confirm whether the Mono Q peaks 3 and 4 correspond to non-covalently associated 23+18 kDa and

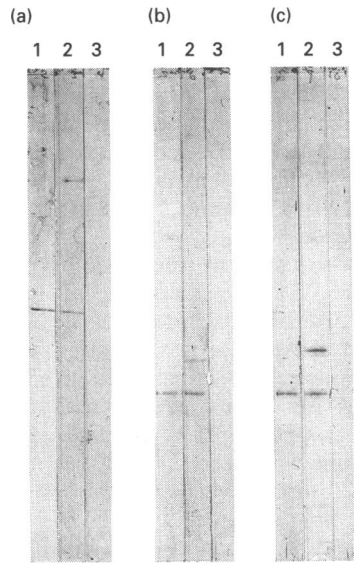

Figure 4 Western blot of calpain and the 'dimers'

(**a**) Calpain; (**b**) 22+18 kDa dimer; (**c**) 23+18 kDa dimer. Lane 1, anti-18 kDa serum; lane 2, anti-calpain serum; lane 3, normal rabbit antiserum.

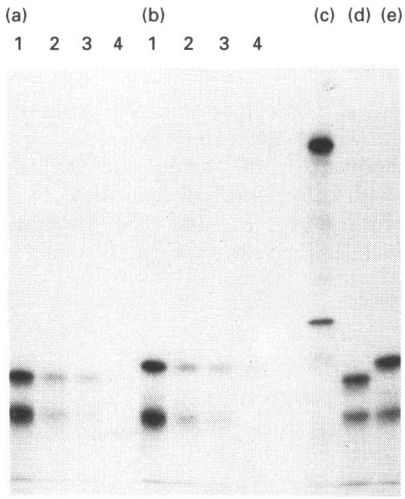

Figure 5 Dimer immunoprecipitations (autoradiograph)

Lane 1, 1:20 anti-18 kDa serum; lane 2, 1:200 anti-18 kDa serum; lane 3, 1:2000 anti-18 kDa serum; lane 4, 1:20 normal rabbit serum. (**a**) 22+18 kDa dimer; (**b**) 23+18 kDa dimer; (**c**)–(**e**) Iodinated proteins prepared using the Bolton and Hunter reagent; (**c**) iodinated calpain; (**d**) iodinated 22+18 kDa dimer; (**e**) iodinated 23+18 kDa dimer. For experimental details, see the Results section.

22+18 kDa heterodimers, the samples were analysed by gel filtration on an h.p.l.c. TSK G2000SW column. The 'dimers' were eluted as single peaks with molecular mass approx. 43 kDa. Samples of calpain and calpain autolysed in the presence of 5 mM Ca^{2+} for 15 min were also run. The 110 kDa calpain peak was converted predominantly into a 43 kDa peak by autolysis. Analysis of this peak by SDS/PAGE demonstrated that it contained the 42 kDa active-site domain fragment of calpain and the dimers. Thus the dimer species are present after only 15 min of autolysis, suggesting that they are representative of a structure that is present in native calpain.

Further evidence that the Mono Q peaks 3 and 4 correspond to non-covalently associated 23+18 kDa and 22+18 kDa heterodimers was obtained from immunoprecipitation experiments. The Western blots shown in Figure 4 demonstrate that the anti-(m-calpain) serum reacts with both subunits of calpain and with both polypeptides of the dimers, whereas the anti-18 kDa serum only reacts with the calpain small subunit and with the 18 kDa polypeptide of the dimers. The two dimer species were radioiodinated using the Bolton and Hunter reagent and the protocol supplied by Amersham International plc. Samples of each dimer (100000 c.p.m.) were incubated for 1 h on ice in 100 μl of 20 mM Tris/HCl, pH 7.5, containing 137 mM NaCl, 5 mM EDTA, 1 mM PMSF, 100 μg/ml aprotinin, 0.1% Tween 20 and dilutions of the anti-18 kDa serum or normal rabbit serum. Sepharose CL-6B (10 μl of a 50% suspension) was added and the samples were mixed for 1 h at 4 °C. The samples were centrifuged, Protein A–Sepharose (10 μl of a 50% suspension) was added to the sample supernatants, and mixing was continued for 1 h at 4 °C. The samples were then spun, and the Protein A–Sepharose was washed with 3 × 300 μl of buffer. Gel sample buffer (40 μl) was added to the washed Protein A–Sepharose and the eluted proteins were analysed by SDS/PAGE followed by autoradiography. The results are shown in Figure 5 and demonstrate that both polypeptides of the dimers are immunoprecipitated by antiserum reacting against only the 18 kDa polypeptide.

Autolytic fragments similar to those described in the present paper were observed by Nishimura and Goll (1991) for bovine skeletal-muscle m-calpain. These authors also concluded that the Ca^{2+}-binding domains were non-covalently associated, based solely on gel-filtration experiments. Imajoh et al. (1987) have reported that carboxypeptidase digestion of calpain subunits prevents reconstitution of the subunits into proteolytically active heterodimers. They concluded that the C-terminal portions of the subunits are important for subunit association. The difference in molecular mass between the 22 and 23 kDa peptides observed on SDS/PAGE appears greater than expected for peptides differing by only five amino acids at the N-terminus, perhaps indicating a difference in the C-terminus of the peptides. Therefore, in the light of the observations of Imajoh et al. (1987), characterization of the C-termini of the dimers prepared by prolonged autolysis was undertaken. This point was not pursued by Nishimura and Goll (1991). Various attempts were made to determine the C-terminal sequences of the 18 kDa, 23 kDa and 22 kDa polypeptides, but this was only partially successful. The samples were therefore analysed by electrospray m.s. The 18 kDa polypeptide had a molecular mass of 20264.5±2.9 Da. The theoretical mass calculated from the amino acid sequence is 20263 Da, thus the isolated 18 kDa polypeptide extends to the C-terminus of the small subunit. The 23 kDa and 22 kDa polypeptides had masses of 23093.8±1.8 Da and 22551.4±1.7 Da respectively. The difference between these masses is 542.4±3.5 Da. The difference expected from the difference in N-terminal sequence (SEKKA) is 542.7 Da. Thus the 23 kDa and 22 kDa peptides have the same C-terminus. The theoretical masses of polypeptides from human and rabbit m-calpain, homologous to the 23 kDa peptide and extending to the C-termini, are 22915 Da and 22865 Da respectively. It is therefore highly likely that the 23 kDa and 22 kDa pig polypeptides extend to the C-terminus of the large subunit. The structures of the isolated dimers are therefore consistent with the

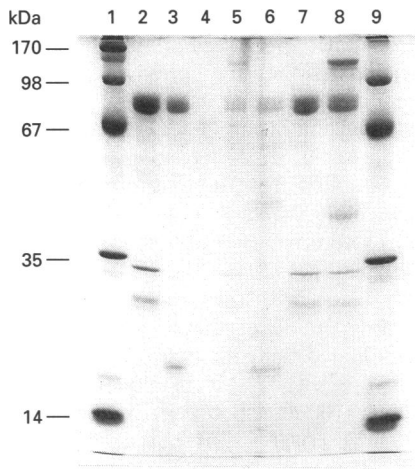

Figure 6 Calpain binding to calpastatin–Sepharose

SDS/PAGE (12.5% gels) of samples from the binding assay described in the Experimental section. Lanes 1 and 9, molecular-mass markers; lane 2, calpain standard; lane 3, protein bound from incubation of calpain, calpastatin–Sepharose and Ca^{2+}; lane 6, supernatant from lane 3; lane 4, protein bound from incubation of calpain, calpastatin–Sepharose and EDTA; lane 7, supernatant from lane 4; lane 5, protein bound from incubation of calpain, calpastatin–Sepharose, Ca^{2+} and calpastatin in solution; lane 8, supernatant from lane 5.

proposal by Imajoh et al. (1987) that the C-terminal portions of the subunits are important for association.

The fragments of calpain generated under non-denaturing conditions by prolonged autolysis as described above, the isolated calpain subunits and active-site-blocked calpain were analysed for their ability to bind to calpastatin–Sepharose. The results are shown in Figures 6 and 7. Figure 6 characterizes the binding assay and shows that calpain binding to calpastatin–Sepharose is Ca^{2+}-dependent and can be competed for by calpastatin in solution. The calpain bound to calpastatin–Sepharose is largely converted into the 80+18 kDa form. Presumably the initial rapid autolytic events occur before binding of calpain to the calpastatin–Sepharose. Figure 7 shows that calpain (80+30 kDa and 80+18 kDa forms), E64-blocked calpain and iodoacetic acid-blocked calpain bind to calpastatin–Sepharose, whereas the 80 kDa large subunit, the 42 kDa active-site fragment, the 18 kDa small-subunit Ca^{2+}-binding domain and the two Ca^{2+}-binding domain dimers (22+18 kDa and 23+18 kDa) do not bind to calpastatin–Sepharose.

In order to assess the relative affinities of calpain and active-site-blocked calpain for calpastatin, the following experiment was carried out. Calpain (0.23 μM) was incubated with calpastatin (0.23 μM active sites, just sufficient to inhibit all the calpain present), iodoacetic acid-blocked calpain (11.4 μM) and azocasein (254 μM) under the conditions used for the azocasein assay (100 mM Tris/HCl, pH 7.5, 100 mM KCl, 1 mM 2-mercaptoethanol and 5 mM excess Ca^{2+}). The resultant enzyme activity was 0.36 absorbance units (S.E.M. = 0.05, $n = 4$). The activity of the enzyme in control experiments of calpain with blocked calpain and no calpastatin, or calpain alone was 0.78 absorbance units (S.E.M. = 0.02, $n = 4$), and of calpain with calpastatin was 0.04 absorbance units (S.E.M. = 0.03, $n = 4$). Thus the presence of the blocked enzyme prevents complete inhibition of calpain by calpastatin. It is possible to calculate the dissociation constant for blocked calpain binding to calpastatin (K_{iB}) from these experiments. It was assumed that the following equilibria exist in the incubation mixture:

$$E + S \rightleftharpoons ES$$

$$E + I \rightleftharpoons EI$$

$$B + I \rightleftharpoons BI$$

where E is calpain, I is calpastatin, S is azocasein and B is blocked calpain. Using the expressions relating the concentrations of components to the various equilibrium dissociation constants, and with known total concentrations of E, I, S and B, and known values of K_m, K_{iE} and [ES] (calculated from the experimental data, [ES] = velocity/$k_{cat.}$), K_{iB} could be calculated

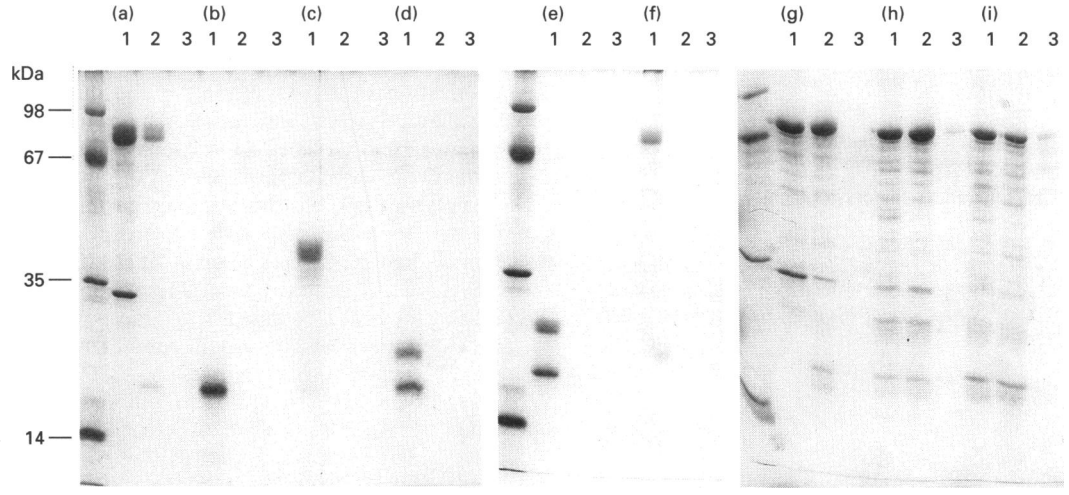

Figure 7 Calpain fragments and active-site-blocked calpain binding to calpastatin–Sepharose

SDS/PAGE (12.5% gels) of samples from the binding assay described in the Experimental section. Lane 1, protein tested; lane 2, protein bound to calpastatin–Sepharose; lane 3, protein bound to control Sepharose. (**a**) Calpain; (**b**) 18 kDa fragment; (**c**) 42 kDa fragment; (**d**) 22+18 kDa dimer; (**e**) 23+18 kDa dimer; (**f**) 80 kDa subunit; (**g**) calpain; (**h**) E64-blocked calpain; (**i**) iodoacetic acid-blocked calpain.

by iterative solution of simultaneous equations based on the procedure of Storer and Cornish-Bowden (1976). It was found that $K_{iB} = 1.5 \times 10^{-7}$ M when K_{iE} was set to 10^{-9} M (Maki et al., 1988) and $K_{iB} = 1.5 \times 10^{-8}$ M if $K_{iE} = 10^{-10}$ M.

DISCUSSION

One of the aims of this paper was to reconsider the question of whether calpain (80 + 30 kDa) is an inactive proenzyme. Calpains are Ca^{2+}-dependent enzymes and it is universally accepted that Ca^{2+} binding is required for expression of enzyme activity. The next issue is whether the Ca^{2+}-bound 80 + 30 kDa form of the enzyme is active, or whether subsequent autolysis is required for the enzyme to be active. The evidence that the Ca^{2+}-bound 80 + 30 kDa enzyme is active (i.e. not a proenzyme) is as follows. First, calpain cleaves itself during autolysis. Secondly the data in this paper show that calpain can react with E64 before autolysis. An additional significance of the result with E64 is that it shows that a molecule the size of E64 has access to the active site in 80 + 30 kDa calpain, and that the site is not blocked by a part of the calpain molecule that is cleaved off during autolysis, as suggested by Suzuki et al. (1987). The remaining issue is whether the Ca^{2+}-bound 80 + 30 kDa enzyme is active towards protein substrates before autolysis. As autolysis proceeds so rapidly following Ca^{2+} binding, it is difficult to design experiments to examine this last issue. The evidence that the Ca^{2+}-bound 80 + 30 kDa enzyme is a proenzyme, in the sense that it cannot react with protein substrates before autolysis, comes from experiments where the time course of substrate degradation were determined at various Ca^{2+} concentrations, and a lag phase observed at low Ca^{2+} (Coolican et al., 1986; DeMartino et al., 1986). At low Ca^{2+} levels, the rate of autolysis is slowed and the lag is interpreted as the time required for the conversion of the 80 + 30 kDa proenzyme into the autolysed active enzyme. However, the results are also consistent with the 80 + 30 kDa form being active towards protein substrates if the following interpretation of the lag is adopted. The effect of low Ca^{2+} is simply to reduce the number of active (Ca^{2+}-bound) 80 + 30 kDa molecules in the mixture and hence decrease the rate of substrate turnover. As these 80 + 30 kDa autolyse they produce molecules which will remain active because they have higher Ca^{2+} affinity (a greater proportion will remain in the active Ca^{2+}-bound state), thus the rate of substrate turnover increases. Thus the observed lag phase does not provide evidence that the 80 + 30 kDa species is a proenzyme unable to turn over substrate. Indeed, Cong et al. (1989), who studied the Ca^{2+}-dependence of autolysis and proteolysis, stated that their results did not demonstrate that unautolysed m-calpain is inactive. On the basis of these various observations there seems no compelling reason to consider calpain as a proenzyme.

Prolonged autolysis of m-calpain generated a 42 kDa active-site-containing fragment, a C-terminal fragment of the small subunit and two dimer species (Figure 2). Sequence analysis of the autolysis products showed that the two polypeptides in the dimers include the C-terminal Ca^{2+}-binding domains of the two calpain subunits (Figure 3c). The existence of these dimers suggests that non-covalent interaction between the two C-terminal Ca^{2+}-binding domains of the calpain subunits is a major site of subunit contact in the whole molecule, as proposed for bovine skeletal muscle m-calpain by Nishimura and Goll (1991). Interestingly, neither of the active-site-containing fragments of calpain described in the present paper (isolated large subunit or 42 kDa fragment) showed proteolytic activity. Isolation of the 80 kDa subunit involves the use of 1 M NaSCN. (Whether this causes any denaturation is uncertain; the 18 kDa polypeptide prepared using the same technique is functional in Ca^{2+} binding, suggesting that no denaturation occurs.) The 42 kDa fragment is purified under non-denaturing conditions. The calpastatin–Sepharose experiments showed that only calpain (80 + 30 kDa and 80 + 18 kDa forms) will bind to calpastatin, and not the isolated active-site-containing 80 kDa subunit or the 42 kDa active-site fragment (Figure 7). It appears that the active-site domain does not show the properties of a functional site in isolation from the rest of the molecule.

Analysis of the effect of active-site-blocked calpain on the inhibition of calpain by calpastatin shows that active-site-blocked calpain has an affinity for calpastatin about two orders of magnitude lower than the native enzyme. However, the binding of active-site-blocked calpain to calpastatin observed in the calpastatin–Sepharose binding assay (Figure 7) shows that considerable binding affinity remains between calpain and calpastatin even in the presence of E64 or iodoacetic acid. Thus binding of calpain to calpastatin involves both residues not occupied by low-molecular-mass inhibitors, as suggested previously (Cottin et al., 1983; Kapprell and Goll, 1989; Kawasaki et al., 1989), and the active-site residues. A relatively large area of interaction is expected for an enzyme–inhibitor complex with tight-binding characteristics and great specificity.

Nishimura and Goll (1991) analysed the binding of calpain (from bovine skeletal muscle) and calpain autolytic fragments to a calpastatin–Sepharose affinity column. In agreement with the data presented here, they found that the isolated 80 kDa subunit of m-calpain and an active-site-containing autolytic fragment did not bind to the column. However, in contrast with the results presented here, they did observe binding of the autolytically derived Ca^{2+}-binding domains and the isolated small subunit. The experimental conditions used by Nishimura and Goll (1991) were somewhat different from those described here. The affinity column was made by coupling calpastatin to 2,2,2-trifluoroethanesulphonyl-activated Sepharose 4B. Binding was assessed by running the protein sample through the column rather than by binding to the affinity matrix in suspension followed by three washing steps. The column buffer used by Nishimura and Goll (1991) was 20 mM Tris/HCl, pH 7.5, containing 2 mM $CaCl_2$ and 14 mM 2-mercaptoethanol. The difference in results may be because the procedure used by Nishimura and Goll (1991) allows for the detection of weaker affinity interactions between calpain fragments and calpastatin. Another possibility is that the differences arise because of the lower solubility of calpain and its fragments in Ca^{2+} at low ionic strengths (Crawford et al., 1990).

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