The effects of autolysis on the structure of chicken calpain II

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When chicken calpain II autolysed in the presence of Ca^{2+} , it underwent limited proteolysis to give peptides of M_r 54000 and 37000, and several of M_r approx. 30000 and 18000. The autolytic peptides were purified and their *N*-terminal amino acid sequences determined. By comparison of these sequences with the known sequence of the complete calpain molecule, the autolytic cleavage sites were identified. The structural integrity of the molecule during autolysis was investigated by gel-permeation chromatography. Experiments were also done to test the reversibility of adding EDTA to calpain during autolysis, measured as recoverable enzyme activity assayed in the presence of Ca^{2+} . The results are presented in terms of a model for the structural changes occurring in calpain during autolysis. It was concluded that the loss of enzymic activity, which is a consequence of autolysis, was due to dissociation of the autolytic peptides after cleavage of the calpain large subunit within the third domain.

INTRODUCTION

Calpains are Ca^{2+} -dependent cysteine proteinases that are found in the cytoplasm of animal cells. The physiological roles of calpains are not well defined, but the enzymes have been implicated in diverse situations, including cytoskeletal modification during platelet activations (Fox *et al.*, 1985), regulation of hippocampal glutamate receptors (Baudry & Lynch, 1980), regulation of protein kinase C (Melloni *et al.*, 1986), neurofilament modification during axonal transport (Schlaepfer *et al.*, 1985) and muscle protein degradation after injury or Ca^{2+} influx (Furuno & Goldberg, 1986). Calpains have two subunits: one of M_r 80000, which contains a papain-like active-site domain and a C-terminal calmodulin-like Ca^{2+} -binding domain (Ohno *et al.*, 1984), and one of M_r 30000, which contains an *N*-terminal glycine-rich domain and a C-terminal calmodulin-like Ca^{2+} -binding domain (Sakihama *et al.*, 1985). Two types of calpains are well characterized: calpains I, which are active at micromolar concentrations of Ca^{2+} , and calpains II, which are active at millimolar concentrations of Ca^{2+} *in vitro*. The two types have identical 30000- M_r subunits, but distinct 80000- M_r subunits (Yumoto *et al.*, 1984; Kawasaki *et al.*, 1986).

Calpains autolyse in the presence of Ca^{2+} . The first cleavages, which occur very rapidly after the addition of Ca^{2+} to the enzyme, convert the 30000- M_r subunit into an 18000- M_r form and convert the 80000- M_r subunit into a 78000- M_r form (Suzuki *et al.*, 1981b; Dayton, 1982; Parkes *et al.*, 1985). These events increase the Ca^{2+} -sensitivity of the enzyme (Suzuki *et al.*, 1981a; Hathaway *et al.*, 1982); however, it is not clear which cleavage is responsible for this (Coolican *et al.*, 1986; Imajoh *et al.*, 1986b; DeMartino *et al.*, 1986). Continued incubation in the presence of Ca^{2+} results in further autolysis of the large subunit and loss of enzymic activity (Suzuki *et al.*, 1981b; Mellgren *et al.*, 1982). For chicken calpain II the large subunit is degraded, over 2 h at room

temperature, to active-site-containing fragments of M_r 54000 and 37000, after which no further significant autolysis occurs. The loss of enzyme activity proceeds much more slowly than the cleavages in the large subunit (Parkes *et al.*, 1985).

In order to gain a detailed understanding of autolysis, it is necessary to define the structural changes occurring in the enzyme and to determine the event which results in loss of enzyme activity. The present paper describes the isolation and *N*-terminal-amino-acid-sequence analysis of the autolytic peptides of calpain and experiments designed to elucidate what causes loss of enzyme activity. The results are discussed in relation to a model for the changes occurring in calpain subsequent to Ca^{2+} binding.

EXPERIMENTAL

Materials

E-64 was from Sigma Chemical Co., Poole, Dorset, U.K. Suc-Leu-Tyr-NHMec was from Bachem (U.K.), Saffron Walden, Essex, U.K., and was made up as a 25 mM stock solution in dimethyl sulphoxide. Azocasein was prepared as described by Barrett & Kirschke (1981). [^3H]Ac-Ep-459 was prepared as described by Parkes *et al.* (1985).

Methods

Preparation of calpain. Calpain II was prepared from chicken gizzard smooth muscle and the active-site concentration determined by titration with E-64 as described previously (Parkes *et al.*, 1985). The protein concentration of calpain solutions was determined from the A_{280} , by using $A_{280}^{1\%} = 14.0$ (Sugita *et al.*, 1980; Tsuji & Imahori, 1981) and an M_r value of 110000.

Assay for calpain. For the assays with azocasein as a substrate, calpain was incubated in 0.5 ml of 100 mM-Tris/acetic acid buffer, pH 7.5, containing 100 mM-KCl, 5 mM- CaCl_2 and 0.02% (v/v) 2-mercaptoethanol at

Abbreviations used: NHMec, 4-methyl-7-coumarylamide; E-64, L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-guanidino)butane; Ac-Ep-459, L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-acetamido)butane; PTH, phenylthiohydantoin.

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30 °C in the presence of 0.6% azocasein. After 30 min the reaction was stopped by the addition of 0.5 ml of 20% (w/v) trichloroacetic acid. After cooling for 10 min at 4 °C, the sample was centrifuged at 8000 *g* for 1.5 min and the A_{366} of the supernatant was recorded.

Continuous fluorimetric assays for calpain were done in a Perkin-Elmer LS-3 spectrofluorimeter, standardized with 1 μ M-NH₂Mec for readings at $\lambda_{\text{excitation}}$ 360 nm and $\lambda_{\text{emission}}$ 460 nm. The assays were done at 22 °C in 3 ml of 50 mM-Tris/HCl buffer, pH 7.5, containing 5 mM-CaCl₂, 1 mM-dithiothreitol, 0.1% Triton X-100 and 250 μ M-Suc-Leu-Tyr-NHMec as substrate. The assays were started by the addition of enzyme.

Preparation of autolytic peptides and calpain subunits.

Calpain (approx. 6 mg) in 1 ml of 50 mM-Tris/HCl buffer, pH 7.5, containing 50 mM-NaCl, 5 mM-EDTA and 0.1% (v/v) 2-mercaptoethanol was made 5 mM in CaCl₂ (final approximate concentration of free Ca²⁺) and incubated at room temperature for 45 min. The solution was then made 20 mM in EDTA, 5 mM in dithiothreitol and 8 M in guanidinium chloride, and incubated for 2 h at 37 °C. Finally, iodoacetic acid was added (from a 1 M stock solution that had been neutralized with NaOH) to give a final concentration of 100 mM, and the sample incubated on ice for 1 h. The sample was then run on a column (97 cm $\times$ 1.6 cm diameter) of Sephacryl S-300 equilibrated with 50 mM-Tris/HCl buffer, pH 7.5, containing 6 M-guanidinium chloride. Peptide pools were made from fractions from the column, dialysed extensively against 0.1% NH₄HCO₃ and freeze-dried.

The reduced and carboxymethylated calpain subunits were prepared using a similar procedure, but omitting the initial incubation in the presence of Ca²⁺.

Amino acid sequence analysis. Sequencing was performed using one of the following. A Beckman model 890C protein sequencer modified with microprocessor control and cold-trap attachment. Samples were run using a double coupling program with 0.1 M-Quadrol buffer (Beckman program part no. 345802) with only minor modifications. The unstable anilinothiazolinones were converted into stable phenylthiohydantoin (PTH) derivatives by the addition of aq. 25% (w/v) trifluoroacetic acid and incubation at 55 °C for 25 min. All other details were as described by Christie & Gagnon (1982). Alternatively, an Applied Biosystems 470A protein sequencer was run basically as described by Hewick *et al.* (1981). Samples were run using the 02CPTH cycle program (with minor modifications) as included in the Applied Biosystems standard sequencer tape, version 2.0 software (part no. 400355).

Identification of amino acid PTH derivatives was by reverse-phase h.p.l.c. on a Shandon ODS-Hypersil (5 μ m particle size) column (5 mm $\times$ 100 mm) equilibrated in 0.01 M-sodium acetate, pH 4.10, containing 10% (v/v) methanol, and eluted with a linear gradient of ethanol/acetonitrile (1:1, v/v) from 5 to 45% (v/v) in 30 min at 1.0 ml/min. A Waters dual-pump system generated the gradient, connected to a Waters WISP 710B sample processor. The runs were controlled and integrated by a Waters 840 Data and Chromatography Control Station utilizing EXPERT software revision 4.0 (Waters part no. 87609).

Samples for amino acid analysis, containing 5 nmol of norleucine standard, were hydrolysed *in vacuo* with

Fig. 1. Purification of autolytic peptide and calpain subunits

Calpain, with or without autolysis, was reduced and carboxymethylated and chromatographed on Sephacryl S-300 in 50 mM-Tris/HCl buffer, pH 7.5, containing 6 M-guanidinium chloride. (a) Elution profile of the column for autolysed calpain. (b) SDS/10%-(w/v)-polyacrylamide-gel electrophoresis of autolytic peptide pools made as indicated in (a) and of the non-autolysed calpain subunits. Lane 1, 80000-*M_r* subunit; lane 2, 30000-*M_r* subunit; lanes 3–7, peptide pools 1–5; lane 8, *M_r* marker; and lane 9, peptide pool 6.

5.7 M-HCl containing 0.04% (v/v) phenol at 110 °C for 24 h. Samples were then analysed on an LKB Instruments model 4400 amino acid analyser using sodium citrate or lithium citrate buffers.

Electrophoresis. SDS/polyacrylamide-gel electrophoresis was with gels of 10% or 12.5% (w/v) polyacrylamide and the 2-amino-2-methylpropane-1,3-diol discontinuous buffer system described by Bury (1981). The samples were prepared by mixing them with an equal volume of 83 mM-2-amino-2-methylpropane-1,3-diol/HCl buffer, pH 8.4, containing 6 M-urea, 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol, and incubating them in a boiling-water bath for 2 min. The *M_r* markers used were: phosphorylase *a* (*M_r* 98000), transferrin (78000), bovine serum albumin (68000), IgG heavy chain (50000), carbonic anhydrase (29000), soyabean trypsin inhibitor (21000), cytochrome *c* (12750) and aprotinin (6500). The gels were scanned with an RFT Transidyne 2955 scanning densitometer.

Peptide	Amount used (nmol)	Sequence	Initial yield (nmol)	Stepwise yield (%)
No. of residues . . .		1 10 20 30		
80 000- M_r subunit	11.4	No sequence detectable		
30 000- M_r subunit	4.83	7 -LLGGGGGGGGGGGGGLPAGLG-SLGGL ps FLKGGGGGGGGGGGLGGGLGNVLGGL ISGA	2.25	98.2
Pool 1*	5.10	18 AEGVGE-NNAVKYLNQ c1 AEGVGEHNNAVKYLNQ	1.23	93.1
Pool 2*	3.15	18 AEGVGE-NNAVKYLNQ c1 AEGVGEHNNAVKYLNQ	0.60	94.2
Pool 3*	1.25	18 AEGVGE-NNAVKYLNQ c1 AEGVGEHNNAVKYLNQ	0.65	91.8
Pool 4*	1.55	18 AEGVGE-NNAVKYLNQDYEA c1 AEGVGEHNNAVKYLNQDYEA	0.75	93.0
Pool 5*	3.46	88 VDPNESEE ps IEANESEE	0.23	NC
		88 VDPNESEEV-QF--LFTQLA ps IEANESEEVQRFRRLFAQLA	1.14	90.8
		529 -TEDDIEDGFKNMFQQLA c1 ITEDDIEDGFKNMFQQLA	0.48	94.2
Pool 6*	26.0	18 AEGVGE--N c1 AEGVGEHNN	0.37	NC
		88 -DPNESEEV-QF--LFTQ ps IEANESEEVQRFRRLFAQ	0.44	93.0
		529 -QLAGMEVGFKNMFQQLA c1 ITEDDIEDGFKNMFQQLA	0.31	90.2
Pool 3†	33.0	408 ---LVALMQK c1 CSFLVALMQK	0.21	90.8
Pool 4†	34.0	85 SIMVDPNESEEV-QF ps YSNIEANESEEVQRQF	0.18	91.7

Fig. 2. N-Terminal amino acid sequences of autolytic peptides and calpain subunits

The Figure gives the results of N-terminal amino acid sequence analysis of the samples shown in the first column. * and † indicate pools from different Sephacryl S-300 chromatographic runs from separate preparations of calpain autolytic peptides. Multiple sequences were obtained for pools 4, 5 and 6 from the * run, because the peptide pools could not be purified further. For the † run considerable problems of insolubility were encountered. The sequences presented for pools 3 and 4 were the only sequences detected in these pools, but they obviously represent only minor components in the pools. The sequences are shown using the one-letter amino acid code; a gap (–) indicates the residue was unidentified. Below each sequence the corresponding homologous sequence determined by cDNA analysis is given; above each sequence there is a number corresponding to the position of the first amino acid of the peptide in the sequence of the complete subunit. Abbreviations used: ps, data from cDNA sequence analysis of the small subunit of porcine calpain (Sakihama *et al.*, 1985); c1, data from cDNA sequence analysis of the large subunit from chicken skeletal-muscle calpain II (Ohno *et al.*, 1984); NC, not calculable.

Fig. 3. Position of the autolytic peptides of calpain within the complete molecule

The two subunits of calpain are drawn showing the domain boundaries. In the large subunit the active-site domain is represented by residues 80–320. The two Ca^{2+} -binding domains are residues 560–705 of the large subunit and residues 98–266 of the small subunit. The autolytic peptides are positioned to show the cleavage points and are identified by their *N*-terminal sequences (see Fig. 2). * and † indicate pools from different Sephadex S-300 runs.

Liquid-scintillation counting. Radioactivities of samples were counted in a United Technologies Packard CD300 liquid-scintillation counter, with Pico-Fluor 30 scintillant (Packard) and automatic quench correction.

RESULTS

Preparation of autolytic peptides and calpain subunits

Calpain was autolysed in the presence of Ca^{2+} to give the stable mixture of major peptides of M_r 54000 and 37000, and several at approx. 30000 and 18000, which had been observed previously (Parkes *et al.*, 1985). This mixture was reduced, carboxymethylated and then chromatographed on Sephadex S-300 as described under 'Methods'. The elution profile from one Sephadex S-300 column run is shown in Fig. 1(a). Attempts at further purification of the autolytic peptides by reverse-phase or ion-exchange chromatography were unsuccessful, owing to problems of solubilization. Pools (numbered 1–6) were therefore made as indicated in Fig. 1(a) and used directly for amino acid sequence determination. The amount in each pool was found by amino acid analysis. Fig. 1(b) shows SDS/polyacrylamide-gel electrophoresis of samples from these pools, together with samples of the reduced and carboxymethylated calpain subunits, also isolated by chromatography on Sephadex S-300 in buffer containing 6 M-guanidinium chloride.

Amino acid sequence analysis of autolytic peptides and calpain subunits

N-Terminal amino acid sequence analysis of the peptide pools 1–6, and of the calpain subunits, was performed as described under 'Methods'. The results are presented in Fig. 2. Alignment of the sequences obtained with the published sequences for porcine calpain and chicken skeletal-muscle calpain allows the position of the autolytic peptides within the calpain molecule to be defined. This is shown in Figs. 2 and 3. The *C*-terminal position of the peptides was not experimentally determined, but is based on the observed M_r of the peptides and the cleavage points found (see Table 1). The intact large subunit gave no sequence; this is consistent with a report that the *N*-terminus is blocked (Ohno *et al.*, 1984). The data confirm that the 54000- M_r and 37000- M_r peptides contain the active site, as previously reported (Parkes *et al.*, 1985), and that one of the autolytic cleavages in the large subunit is after residue 17 (Ohno *et al.*, 1984). The other autolytic cleavages in the large subunit occur in domain 3 (residues 320–560). The sequence obtained for the small subunit corresponds to residue 7 onwards for the porcine sequence. The *N*-terminus of the small subunits of other calpains are blocked (Sakihama *et al.*, 1985; Kawasaki *et al.*, 1986), thus it is possible that the small subunit analysed here was slightly degraded. The major cleavage in the small

Table 1. M_r values of calpain II autolytic peptides

The calculation of the M_r value is based on the number of residues and an average M_r of 110 for each amino acid. The observed M_r values are found from SDS/polyacrylamide-gel electrophoresis, and are approximate values. The agreement between the calculated and observed M_r values confirms the C-terminal position of the peptides indicated in Fig. 3. Numbered pools are from Sephacryl S-300 chromatography (* and † represent separate runs).

Pool	Peptide		M_r	
	Subunit	Residues	Calculated	Observed
1†	Large	18–528	56 200	54 000
2†	Large	18–407	42 900	37 000
3*	Large	408–705	32 800	32 000
5†	Large	529–705	19 500	18 000
	Small	88–266	19 700	18 000

subunit is after residue 87, giving a C-terminal peptide of M_r 18 000; the N-terminal peptide from this cleavage was not recovered. An alternative is cleavage after residue 84, as evidenced by the isolation of the peptide with sequence SIMVDP (one-letter notation). The presence of the minor peptide with sequence -QLAGMEVGFKN in Sephacryl S-300 A Pool 6, which is not homologous at its N-terminus with the sequence of chicken skeletal-muscle calpain, suggests the existence of more than one type of calpain II in chicken.

Investigation of the dissociation of autolysed calpain

Having defined the positions of the autolytic cleavages, the next stage in analysing the effects of autolysis on the structure of calpain was to look for changes in the structural integrity of the molecule. The first experiments were to analyse, by gel-permeation chromatography, calpain that had been autolysed for different times and to look for dissociation of autolytic fragments. Calpain (1.4 mg) in 50 mM-Tris/HCl buffer, pH 7.5, containing 5 mM-EDTA, 50 mM-NaCl and 0.1% (v/v) monothio-glycerol was incubated for 0, 15 s, 3.5 min or 60 min at 22 °C in the presence of Ca^{2+} (final [free Ca^{2+}] approx. 5 mM). The reaction was stopped by adding EDTA (20 mM final concn.). The mixture was then chromatographed on a column (90 cm $\times$ 1.6 cm diam.) of Sephacryl S-200 equilibrated in 50 mM-Tris/HCl buffer, pH 7.5, containing 5 mM-EDTA. Fractions from the columns were analysed by SDS/polyacrylamide-gel electrophoresis. The results are shown in Fig. 4. The calpain used in these experiments had a partially degraded small subunit [Fig. 4b, gel (i)]. The presence of a 'staircase' of bands suggests that autolysis of the small subunit involves many cleavages of the molecule in the N-terminal region and fragmentation of this region into small peptides. This may explain why no peptide corresponding to the N-terminus of the small subunit was isolated for sequence analysis. After 15 s of autolysis the small subunit was completely converted into the 18 000 M_r form; however, no dissociation of the two subunits was observed. After 3.5 min, cleavage of the large subunit was observed, with the appearance of the band at M_r 54 000. The elution position of the 54 000- M_r band, and some of the

18 000- M_r band from the Sephacryl S-200 column, suggests that, after cleavage of the large subunit in domain 3, the subunits dissociate. After 60 min autolysis the 80 000- M_r subunit was completely cleaved and the peptides were eluted in the positions expected from their individual M_r values, indicative of complete dissociation. The material in the high- M_r peaks from these columns showed enzymic activity, except for the column run after 60 min autolysis, where no activity was recovered.

The possibility that cleavage in domain 3 of the large subunit results in dissociation of the autolytic fragments in the presence of EDTA was further investigated in the following experiment. The experiment was designed to show whether adding EDTA to calpain during autolysis, followed by putting the enzyme back into Ca^{2+} and assaying for enzyme activity, always resulted in full recovery of enzyme activity. Calpain (0.2 mg) in 0.175 ml of 50 mM-Tris/HCl buffer, pH 7.5, containing 5 mM-EDTA, 50 mM-NaCl and 0.1% (v/v) 2-mercaptoethanol was made 5 mM in Ca^{2+} (final approx. [free Ca^{2+}]) and incubated at 22 °C. At various times in the 5 h after addition of Ca^{2+} , samples were removed and assayed for calpain activity with azocasein as the substrate. At various times (2 min, 15 min, 60 min or not at all, in different experiments) EDTA, to give a 20 mM final concentration, was added to the calpain/ Ca^{2+} incubation. The results are shown in Fig. 5(a). In the control experiment (EDTA not added) the activity of calpain decreased gradually over the 5 h, at a much lower rate than the rate of the autolytic cleavages (Fig. 4), as had been observed previously (Parkes *et al.*, 1985). In experiments where EDTA was added, a proportion of the calpain activity was irreversibly lost. The fraction of the total calpain activity remaining decreased at later times of EDTA addition and is represented by the plateau level reached by the curves, say at 2 h.

The structure and reactivity of the calpain at the 2 h time points in the above experiment were examined further as follows. An autolysis experiment was set up under conditions identical with those described above, but in this case samples were removed at various times and analysed by SDS/polyacrylamide-gel electrophoresis. The percentage of intact 80 000- M_r subunit at various times was determined by scanning the gel. The results are shown in Fig. 5(b). The peptide epoxide ^3H -Ac-Ep-459 reacts with the cysteine residue at the active site of enzymically active calpain, giving irreversible inhibition. To examine the ability of partially autolysed calpain to incorporate [^3H]Ac-Ep-459 into the active site, the following was done. An experiment was set up exactly as described above, but with only three calpain samples. EDTA was added at 1 min, 20 min or not at all. At 2 h after the initial addition of Ca^{2+} , the samples were made 5 mM in Ca^{2+} (final approx. [free Ca^{2+}]) and 15 μM in [^3H]Ac-Ep-459 in a total volume of 0.5 ml, and incubated for 30 min at 22 °C. The reaction with ^3H -Ac-Ep-459 was stopped by adding EDTA to a final concentration of 20 mM, and the samples dialysed extensively against 50 mM-Tris/HCl buffer, pH 7.5, containing 5 mM-EDTA. The protein concentration of the samples was then determined from their A_{280} , and the radioactivity incorporated by liquid-scintillation counting. The percentage of molecules that had incorporated [^3H]Ac-Ep-459 was thus calculated, the results being shown in Fig. 5(b). Fig. 5(b) demonstrates that, as autolysis proceeds, there is a close correlation between

the percentage of enzymic activity that is still stable to the addition of EDTA, the percentage of intact 80000- M_r subunit and the percentage of the enzyme still able to incorporate [3H]Ac-Ep-459.

Increase in Ca^{2+} sensitivity of calpain during autolysis

An increase in the Ca^{2+} -sensitivity of calpain during autolysis has been reported by several authors. To confirm that this also occurred with the chicken calpain

used for the experiments described above, the following was done. Calpain (0.04 mg) in 50 mM-Tris/HCl buffer, pH 7.5, containing 5 mM-EDTA, 50 mM-NaCl and 0.1% (v/v) 2-mercaptoethanol was made 10 mM in Ca^{2+} (final approx. [$free\ Ca^{2+}$]) in a total volume of 0.035 ml at 22 °C. At various times the mixture was diluted to 3 ml with the components required for the fluorimetric assay of calpain, giving a final Ca^{2+} concentration of 0.12 mM, and the activity of the enzyme was determined. The

Fig. 4. Analysis of autolysed calpains on Sephacryl S-200

Calpain was autolysed for various times and then chromatographed on Sephacryl S-200 (for details see the text). (a) Elution profiles for calpain autolysed for 0 (i), 15 s (ii), 3.5 min (iii) and 60 min (iv). (b) SDS/12.5%-(w/v)-polyacrylamide-gel electrophoresis of samples taken from fractions across the protein peaks of the column runs shown in (a): (i) no autolysis; (ii) 15 s autolysis; (iii) 3.5 min autolysis; and (iv) 60 min autolysis.

results are given in Fig. 6 and show that the activity of the enzyme at the low Ca^{2+} concentration was increased by preincubation at 10 mM- Ca^{2+} , when autolysis is assumed to occur. Longer incubation at 10 mM- Ca^{2+} was inhibitory, probably due to more extensive autolysis and dissociation upon dilution.

Assay of the activity of calpain in the absence of Ca^{2+} during prolonged autolysis, for example during the experiment described in Fig. 5, never reveals any enzymic

activity. Thus autolysis does not produce Ca^{2+} -independent forms of the enzyme.

DISCUSSION

The proposed sequence of events occurring when calpain binds Ca^{2+} is shown in Scheme 1. Ca^{2+} binding activates the enzyme and initiates autolysis. The enzyme is shown as being active before autolysis, since a

Fig. 5. Stability of calpain activity during autolysis

(a) Calpain was incubated in the presence of Ca^{2+} , and at various times samples were taken and assayed for enzymic activity. EDTA was added to the calpain incubations at various times in different experiments as follows: at 2 min (●), at 15 min (□), at 60 min (■) and not at all (○). For details, see the text. (b) The plateau levels of enzymic activity at 2 h from (a) are plotted as a percentage of initial enzymic activity remaining against the time that the EDTA was added to the calpain- Ca^{2+} incubations (i.e. the time autolysis was allowed to proceed) (●). In a separate experiment the percentage of molecules incorporating [^3H]Ac-Ep-459 (analysed at 2 h) after incubation of calpain with Ca^{2+} and addition of EDTA at 1 min or 20 min was also calculated (□). In another experiment the percentage of 80000- M_r subunit remaining intact at various times during autolysis under identical conditions was also determined (○). For details, see the text.

functional active site is required to carry out the autolytic cleavages. However, it has been suggested that, before autolysis, calpain will not act on exogenous substrates (Coolican *et al.*, 1986; DeMartino *et al.*, 1986), thus the initial autolytic cleavages would be intramolecular, in contrast with the later ones, which are intermolecular (Parkes *et al.*, 1985). The first autolytic cleavages are after residue 87 of the small subunit and after residue 17 of the large subunit. A 78000+18000- M_r form of

Fig. 6. Change in Ca^{2+} -sensitivity of calpain during autolysis

Calpain was preincubated in 10 mM- Ca^{2+} for various times and assayed for enzyme activity at 0.12 mM- Ca^{2+} using Suc-Leu-Tyr-NHMeC as substrate. For details, see the text.

calpain, distinct from the 80000+18000- M_r form, has not been identified in the present work, although it has been described by others (Suzuki *et al.*, 1981b; Hathaway *et al.*, 1982). The reason for this may be that the 80000- and 78000- M_r subunits are not resolved on the SDS/polyacrylamide gels used in the present work. Autolytic cleavage after residue 17 of the large subunit certainly occurs (Fig. 2), but the timing of this cleavage is uncertain from the data presented here. The two *N*-terminal fragments produced by these initial cleavages have not been isolated, so it is not known whether they remain associated with the rest of the molecule (as shown) or whether they dissociate. As mentioned in the Results section, it is probable that multiple cleavages occur in the *N*-terminal domain of the small subunit, in agreement with DeMartino *et al.* (1986).

The next step in autolysis is cleavage in domain 3 of the large subunit. This can occur at at least two sites: after residue 407 or after residue 528; these appear to be alternative breakdown pathways (Parkes *et al.*, 1985). Cleavage in domain 3 of the large subunit has profound consequences for the activity of the enzyme. Firstly, after cleavage has occurred in this region of the molecule, dissociation of the autolytic fragments occurs when the molecule is gel-filtered in the presence of EDTA (Fig. 4). It has been reported that incubation of human erythrocyte calpain I with 1 mM- CaCl_2 for 5 min resulted in dissociation of the subunits (Melloni *et al.*, 1982); however, the subunits were separated by gel-permeation chromatography in the presence of Ca^{2+} , so it cannot be concluded that dissociation occurred rapidly. Calpain has been demonstrated to be a heterodimer by cross-linking in the presence of Ca^{2+} (Hatanaka *et al.*, 1985); this is evidence that dissociation is not an early event in autolysis. The second consequence of cleavage in domain 3 is that enzymic activity cannot be recovered once the molecule has been treated with EDTA (Fig. 5). As a consequence of these observations, it is proposed that the slow loss of enzymic activity observed when calpain is incubated in the presence of Ca^{2+} is due to the dissociation of the autolysed enzyme, and that autolytic cleavage

Scheme 1. Effects of autolysis on the structure of chicken calpain II

The Scheme depicts the sequence of events proposed to occur when Ca^{2+} binds to chicken calpain II. Ca^{2+} is shown binding to each subunit; however, it is not intended to convey that this necessarily occurs, nor is the number of Ca^{2+} ions involved indicated.

within domain 3 of the large subunit is a prerequisite for this to occur. Thus loss of enzymic activity is not the immediate result of a proteolytic cleavage.

A model to explain the early events in calpain autolysis, relating to how the enzyme might be activated *in vivo*, has fairly recently been proposed (Imajoh *et al.*, 1986b). The model suggests a sequence of events in which calpain interacts with membranes, undergoes the first two autolytic cleavages and is released into the cytoplasm as a $78\,000 + 18\,000\text{-}M_r$ dimer, with greater Ca^{2+} -sensitivity than the initial enzyme. The model thus incorporates the idea that phospholipid binding to the *N*-terminal domain of the small subunit (Coolican & Hathaway, 1984; Pontremoli *et al.*, 1985; Imajoh *et al.*, 1986a) and autolysis of at least one of the subunits of calpain (Coolican *et al.*, 1986; DeMartino *et al.*, 1986; Imajoh *et al.*, 1986b) increase the Ca^{2+} -sensitivity of the enzyme. The events depicted in Scheme 1 develop this model, providing information on what could happen to the $78\,000 + 18\,000\text{-}M_r$ form of calpain.

Amino acid sequence information is available for the small subunits of porcine, human and rabbit calpains (Sakihama *et al.*, 1985; Ohno *et al.*, 1986; Emori *et al.*,

1986). The sequences are very similar, with the human and rabbit sequences showing 92% homology. Comparison of the sequences for peptides from the chicken small subunit with the porcine sequence (Fig. 2) suggests that the chicken sequence will not be as closely homologous with the group of known sequences as they are with each other. This is expected, since chickens do not show a close evolutionary relationship to the other species.

The sites of autolytic cleavage should give some insights into the substrate specificity of calpains. Work on peptide inhibitors and substrates for calpain has suggested that the enzyme cleaves most readily after the sequence L-L/V-X, where X is F, Y, K or M (Sasaki *et al.*, 1984, 1986). The autolytic cleavages in the large subunit occur in the middle of the octapeptides DRLRAEGV, DEVACSFL and DEEEITED, and one of the cleavages in the small subunit occurs in the middle of SIMVDP. Thus the specificity observed with peptides is not upheld for the autolytic cleavages. This is not surprising, since both the conformation of a native protein and its primary sequence contribute to determining which bonds are susceptible to proteolytic

cleavage. A study of the calpain cleavage sites in the oxidized insulin B-chain also showed a range of specificities (Ishiura *et al.*, 1979). Thus it is difficult to predict the sites at which calpain might cleave any of its physiological substrates.

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