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Calpain inhibition by peptide epoxides

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A Ca^{2+}-activated cysteine proteinase (calpain II) was purified from chicken gizzard smooth muscle by use of isoelectric precipitation, $(NH_4)_2SO_4$ fractionation, chromatography on DEAE-Sepharose CL-6B, Reactive-Red 120–agarose and Mono Q. The apparent second-order rate constants for the inactivation of calpain by a series of structural analogues of L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-guanidino)butane (E-64) were determined. The fastest rate of inactivation was observed with L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-benzyloxycarbonylamino)butane. It was possible to determine the active-site molarity of solutions of calpain by titration with E-64.When incubated with Ca^{2+}, calpain underwent several steps of intermolecular limited proteolysis, via multiple pathways, followed by a slower loss of enzymic activity. The proteolytic steps preceding the loss of activity did not affect the rates of reaction of calpain with E-64 analogues.

Ca^{2+}-activated cysteine proteinases (calpains, EC 3.4.22.17) have been isolated from a wide variety of mammalian and avian tissues (Goll *et al.*, 1983; Murachi, 1983). The enzymes are composed of two subunits of M_r 80000 and 30000. The active site shows homology with papain and is in the 80000-M_r subunit (Suzuki *et al.*, 1983; Ohno *et al.*, 1984). Calpains are maximally active at pH 7.0–8.5 (Murachi, 1983). There are two types of calpain, one fully active at Ca^{2+} concentrations below 1 mM (calpains I) and the other requiring at least 1 mM-Ca^{2+} (calpains II) for full activity (Mellgren, 1980; Kishimoto *et al.*, 1981). The forms have apparently identical 30000-M_r subunits, but different 80000-M_r subunits (Wheelock, 1982; Sasaki *et al.*, 1983). Although calpains show an absolute requirement for Ca^{2+} for activity, they also undergo autolysis in the presence of Ca^{2+}, with eventual loss of enzymic activity (Suzuki *et al.*, 1981*a*,*b*).

The physiological roles of the calpains are not understood, but preparations have been shown to degrade myofibrillar proteins (Dayton *et al.*, 1976), neurofilament proteins (Zimmerman & Schlaepfer, 1982; Kamakura *et al.*, 1983), intermediate-filament proteins (Nelson & Traub, 1982), lens α-crystallin (Yoshida *et al.*, 1984), receptor proteins (Vedeckis *et al.*, 1980; Yeaton *et al.*, 1983; Murayama *et al.*, 1984), microtubule-associated proteins (Klein *et al.*, 1981), membrane proteins (Pant *et al.*, 1983; McGowan *et al.*, 1983) and several kinases (Huston & Krebs, 1968; Takai *et al.*, 1977; Kishimoto *et al.*, 1983). Little is known about the substrate specificity of calpain, except the sites of cleavage of a few peptides (Ishiura *et al.*, 1979; Hirao & Takahashi, 1984). The enzyme is often assayed by measuring the degradation of casein (Waxman, 1981), but more recently fluorogenic peptide substrates have been described (Sasaki *et al.*, 1984).

L-3-Carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-guanidino)butane (E-64) and its ana-

Abbreviations were as follows. The names of amino acids, peptides and their derivatives are abbreviated in accordance with IUPAC–IUB recommendations [*Biochem. J.* (1984) **219**, 345–373]. Additional abbreviations are: f.p.l.c; fast protein liquid chromatography (Pharmacia system). The inhibitors are: E-64, L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-guanidino)butane (also D-isomer where indicated); Ep-420, DL-3-benzoxy-*trans*-2,3-epoxypropionylisoleucyltyrosine methyl ester; Ep-459, L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-amino)butane; Ac-Ep-459, L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-acetamido)butane; Ep-460, L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-benzyloxycarbonylamino)butane; Ep-475, L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(3-methyl)butane; Ep-479, L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(7-amino)heptane; DC-11, L-but-2-ene-*trans*-1,4-dioyl-leucylamido-(4-guanidino)butane.

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logues are potent active-site-directed inactivators of papain and the lysosomal cysteine proteinases (Barrett *et al.*, 1982). Calpain has been shown to be inactivated by E-64 (Sugita *et al.*, 1980). Thus the various epoxide inhibitors are potentially useful in the investigation of the functions of calpain *in vivo*. It was therefore of interest to determine the rates of inactivation of calpain by E-64 and its analogues, and to compare the selectivity of the inhibitors for calpain as opposed to other cysteine proteinases. The present paper describes such experiments with calpain purified from chicken gizzard smooth muscle, a rich source of the enzyme. Since calpain autolysed under the conditions used for inactivation by the epoxide derivatives, the effect of autolysis on the enzyme structure, activity and reactivity with the inhibitors was also investigated. The effect of autolysis on the activity of calpain has wider significance, since the enzyme may autolyse when it is active *in vivo*.

Experimental

Materials

E-64 [as L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)butane] was from Sigma Chemical Co. Poole, Dorset, U.K. The other epoxide inhibitors were a gift from Dr. Kazunori Hanada, Taisho Pharmaceutical Co., Japan, and were as described by Barrett *et al.* (1982), except for [^{3}H]Ac-Ep-459 and Ac-Ep-459, which were prepared as described below. Z-Phe-Ala-CHN_2, Z-Phe-Phe-CHN_2 and Pro-Phe-Arg-CH_2Cl were kindly given by Dr. Elliott Shaw, Brookhaven National Laboratory, Brookhaven, NY, U.S.A. Azocasein was prepared from casein as described by Barrett & Kirschke (1981).

DEAE-Sepharose CL-6B, Sephadex G-25 (medium grade) and the f.p.l.c. Mono Q HR10/10 system were from Pharmacia, Hounslow, Middx., U.K. Reactive-Red 120–agarose was from Sigma Chemical Co.

The chicken gizzards were supplied by G. W. Padley, Bury St. Edmunds, Suffolk, U.K. Papain was from Sigma Chemical Co. [^{3}H]Acetic anhydride (type TRK.2) was from Amersham International, Amersham, Bucks., U.K. Other reagents were of analytical grade and supplied by Fisons, Loughborough, Leics., U.K. or Sigma Chemical Co.

Purification of calpain

Chicken gizzard smooth muscle (1 kg), either fresh or frozen and thawed, was minced and homogenized, with a Waring blender, in 2.5 litres of 50mM-Tris/acetate buffer, pH 8.0, containing 4mM-EDTA and 1mM phenylmethanesulphonyl fluoride. This and subsequent steps were performed at 4°C. The homogenate was spun at 4100*g* for 15min, and the supernatant was filtered through glass-wool. The supernatant was adjusted to pH 6.2 with acetic acid, stirred for 15min, and centrifuged at 4100*g* for 30min. $(NH_4)_2SO_4$ was added to the supernatant to give a 60%-saturated solution, and this was stirred for 30min. The pellet obtained after centrifugation at 4100*g* for 30min was redissolved in 200ml of 50mM-Tris/HCl buffer, pH 7.5, containing 5mM-EDTA and 0.05% (v/v) 2-mercaptoethanol (buffer A). The pH was adjusted to 7.5 with NaOH, and the sample was dialysed against buffer A.

The dialysed sample was centrifuged at 78000*g* for 60min, and the supernatant was loaded on to a column of DEAE-Sepharose CL-6B (35cm × 3cm diam.) equilibrated in buffer A. The column was washed with 150ml of buffer and then eluted with a 1.2-litre linear gradient of buffer A containing 0.07–0.70M-NaCl. Calpain activity was eluted at approx. 0.3M-NaCl, just after a peak of calpain-inhibitory activity (Johnson *et al.*, 1984).

The active fractions from the anion-exchange column were combined and dialysed against 20mM-Tris/HCl buffer, pH 7.5, containing 5mM-EDTA, 0.5M-NaCl and 0.1% (v/v) 2-mercaptoethanol (buffer B). The sample was loaded on to a column of Reactive-Red 120–agarose (20cm × 1.7cm diam.) equilibrated in buffer B, and chromatographed essentially as described by Hathaway *et al.*, (1982). The column was washed with buffer B until the A_{280} of the eluent was less than 0.01. Calpain was then eluted by washing the column with buffer B containing no NaCl. The calpain-inhibitory activity that was eluted from the DEAE-Sepharose column just before calpain did not bind to Reactive-Red–agarose, and was thus completely separated from calpain at this stage.

The fractions containing calpain from the Reactive-Red–agarose column were combined and dialysed against 20mM-Bistris/HCl buffer, pH 7.0, containing 5mM-EDTA, 0.2M-NaCl, 0.1% (v/v) 2-mercaptoethanol and 0.01% NaN_3 (buffer C). The sample was run at 22°C in approx. 20mg portions on the Mono Q column (anion exchange). The column, equilibrated in buffer C, was loaded at 2ml/min and washed with 30ml of buffer C. The column was then eluted with a 160ml linear gradient of 0.06–0.165M-NaCl in buffer C. Calpain eluted from the Mono Q column was concentrated in an Amicon Diaflow apparatus fitted with a PM10 membrane, and stored at 4°C.

Assay of calpain

Calpain was incubated in 0.5ml of 100mM-Tris/acetate buffer, pH 7.5, containing 100mM-KCl, 5mM-$CaCl_2$ and 0.02% (v/v) 2-mercaptoethanol at 30°C in the presence of 0.6% azocasein.

After 30min the reaction was stopped by the addition of 0.4ml of 20% (w/v) trichloroacetic acid. After cooling for 10min at 4°C, the sample was centrifuged at 8000*g* for 1.5min, and the A_{366} of the supernatant was recorded.

Active-site titration of calpain

Active-site titrations of calpain were done with E-64. A series of incubation mixtures were set up containing calpain (approx. 0.5μM) in 100mM-Tris/acetate buffer, pH7.5, containing 100mM-KCl, 5mM-$CaCl_2$ and 0.02% (v/v) 2-mercaptoethanol, with various concentrations of E-64 (0–0.5μM). After 30min at 22°C, the samples were assayed for calpain. Residual activity was plotted against E-64 concentration. The active-site molarity was taken to be equal to the inhibitor concentration required to give just complete inhibition, as was done previously for cathepsin B (Barrett *et al.*, 1982).

Determination of rate constants for inactivation of calpain by E-64 and its analogues

Incubation mixtures were set up at 22°C containing calpain in 100mM-Tris/acetate buffer, pH7.5, with 100mM-KCl and 0.05% (v/v) 2-mercaptoethanol. $CaCl_2$ (5mM final Ca^{2+} concentration) was then added, followed immediately by inhibitor. The inhibitor solutions were made up in either water or methanol. The methanol had no effect on calpain activity at the concentrations used (2%, v/v, maximum). At various times, samples were removed and diluted at least 10-fold for assay of calpain activity. The initial inhibitor concentration was either equimolar with the enzyme, for the faster-acting inhibitors, or was at least 5 times the enzyme concentration, for the slower inhibitors. The results were analysed to obtain the apparent second-order rate constant for inactivation as described by Barrett *et al.* (1982).

Preparation of [^{3}H]Ac-Ep-459 and Ac-Ep-459

[^{3}H]Ac-Ep-459 was prepared by treating Ep-459 in dry dimethylformamide with 1 equivalent each of [^{3}H]acetic anhydride and triethylamine for 2h at 22°C. A second equivalent of acetic anhydride (unlabelled) and of triethylamine in dimethylformamide was then added, and the reaction continued for a further 1h. The volatile by-products were removed by bulb-to-bulb distillation at reduced pressure, and the product was dissolved in methanol. The product was analysed by silica t.l.c. with methanol/acetic acid/chloroform (25:2:73, by vol.) as solvent. No residual Ep-459 was detectable with ninhydrin, and over 95% of the radioactivity was associated with the product (located with I_2 vapour). The concentration of the solution of product was determined by titration with papain that had itself been titrated with E-64 (Barrett & Kirschke, 1981). The specific radioactivity of the product was 55Ci/mol. Ac-Ep-459 was prepared similarly with unlabelled acetic anhydride.

Preparation of radiolabelled calpain

Calpain (approx. 0.5mg) was incubated for 20min at 22°C with a 10-fold molar excess of [^{3}H]Ac-Ep-459 in 0.75 ml of 50mM-Tris/HCl buffer, pH7.5, containing 0.1M-NaCl, 0.05% (v/v) 2-mercaptoethanol and 5mM-$CaCl_2$. The mixture was run on a column (35cm × 1cm diam.) of Sephadex G-25 in 50mM-Tris/HCl buffer, pH7.5, containing 0.1M-NaCl, 0.05% (v/v) 2-mercaptoethanol and 5mM-EDTA to separate the protein from unincorporated radioactivity. The resultant calpain had no detectable enzymic activity and had incorporated 0.98mol of [^{3}H]Ac-Ep-459/mol (S.E.M. 0.02, $n = 6$).

Electrophoresis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was with gels of 12.5% (w/v) and 7% (w/v) polyacrylamide and the 2-amino-2-methylpropane-1,3-diol discontinuous buffer system described by Bury (1981). The samples were prepared by boiling for 5min in 83mM-2-amino-2-methylpropane-1,3-diol/HCl buffer, pH8.4, containing 6M-urea, 1% (w/v) sodium dodecyl sulphate and 1% (v/v) 2-mercaptoethanol. M_r calibration was with phosphorylase *a* (M_r 94000), transferrin (M_r 78000), bovine serum albumin (M_r 68000), IgG heavy chain (M_r 50000), carbonic anhydrase (M_r 29000), soya-bean trypsin inhibitor (M_r 21000), cytochrome *c* (M_r 12750) and aprotinin (M_r 6500).

For tritium-labelled samples the amount of radioactivity in various regions of the gel was determined as follows. Each sample track was sliced into approximately 2.5mm bands; these were further fragmented and placed in scintillation vials. The samples were incubated overnight at 60°C in 0.4ml of 15% (w/v) H_2O_2/aq. 0.5% (v/v) NH_3. Scintillation fluid (4ml) was then added and the radioactivities of the samples were counted.

Scintillation counting

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

Protein concentration

The protein concentration of calpain solutions was determined from the absorbance at 280nm, by using $A^{1\%}_{280} = 14.0$ (Sugita *et al.*, 1980; Tsuji & Imahori, 1981) and M_r 110000.

Results

Purification of calpain

Calpain was purified from chicken gizzard smooth muscle as described above. Chromatography on Mono Q (Fig. 1) separated the calpain activity into four peaks. The material in peak 1, comprising a 78000-M_r chain and a 28000-M_r chain, was pooled and used as purified calpain. Approx. 15mg was obtained from 1kg of tissue. The enzyme was 95–105% active, on the basis of titration with E-64. The purified calpain required 1–3mM-Ca^{2+} for full activity, and is thus a calpain II. The material in peaks 2, 3 and 4 did not have an intact 28000-M_r chain, and was designated 'degraded calpain'. When tissue that had been frozen was used for the preparation rather than fresh tissue, the calpain activity bound less tightly to the Reactive-Red–agarose column and a greater proportion of 'degraded calpain' was found on the Mono Q column.

Inactivation of calpain by E-64 and its analogues

The apparent second-order rate constants for inactivation of calpain by E-64 and its analogues were determined as described in the Experimental section and are presented in Table 1. The values were independent of the inhibitor concentration. No inactivation was observed in the absence of Ca^{2+}.

Pro-Phe-Arg-CH_2Cl, Z-Phe-Phe-CHN_2 and Z-Phe-Ala-CHN_2 were also tested as inactivators of calpain. Rate constants of less than $10 M^{-1} \cdot s^{-1}$ were found for the diazomethanes. The Pro-Phe-Arg-CH_2Cl did not give linear plots for the logarithm of activity against time, but the initial rate of inactivation was in the range 100–$1000 M^{-1} \cdot s^{-1}$.

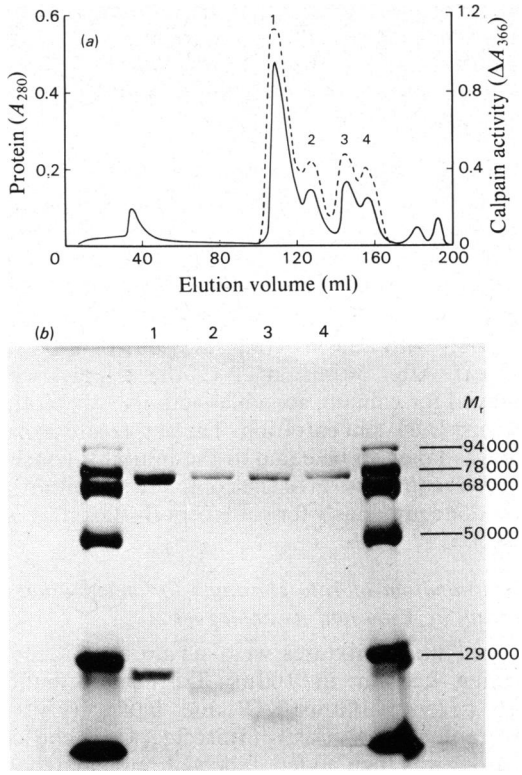

Fig. 1. *Mono Q f.p.l.c. of calpain*
(*a*) The Mono Q HR 10/10 column was loaded with material comprising the active fractions from the Reactive-Red–agarose column in buffer C. The column was washed with 30ml of buffer C and eluted with a linear gradient (160ml) of 0.06–0.165M-NaCl in buffer C. ——, Protein; – – – –, calpain activity. (*b*) The sodium dodecyl sulphate/polyacrylamide-gel electrophoretogram shows samples of the material eluted from the column at the positions marked 1 to 4.

Table 1. *Rate constants for the inactivation of calpain by E-64 and its analogues*
The apparent second-order rate constants for inactivation were determined at 22°C in 100mM-Tris/acetate buffer, pH 7.5, containing 100mM-KCl, 5mM-$CaCl_2$ and 0.05% (v/v) 2-mercaptoethanol as described in the Experimental section. The experiments were performed with either equimolar (+) or a large excess (*) of inhibitor. OH-Fum is L-but-2-ene-*trans*-1,4-dioyl and OH-Eps is L-3-carboxy-*trans*-2,3-epoxypropionyl. The statistical limits are ±S.E.M. ($n = 4$).

Inhibitor	Structure	Rate constant ($M^{-1} \cdot s^{-1}$)
Ep-460+	OH-Eps-Leu-NH-[CH_2]$_4$-NH-Z	23340±480
E-64+	OH-Eps-Leu-NH-[CH_2]$_4$-NH-CH-NH_2-NH	7500±560
Ep-475+	OH-Eps-Leu-NH-CH_2-$CH(CH_3)_2$	7450±340
Ep-479+	OH-Eps-Leu-NH-[CH_2]$_7$$NH_2$	4990±180
Ac-Ep-459+*	OH-Eps-Leu-NH-[CH_2]$_4$-NH-CO-CH_3	3040±130
Ep-459+*	OH-Eps-Leu-NH-[CH_2]$_4$-NH_2	2790±180
E-64 (D)+*	OH-Eps-Leu-NH-[CH_2]$_4$-NH-CH-NH_2-NH	1070±110
Ep-420*	Bzl-DL-Eps-Ile-Tyr-OMe	440± 23
DC-11*	OH-Fum-Leu-NH-[CH_2]$_2$-$CH(CH_3)_2$	6± 3

Table 2. *Rate constants for the inactivation of various forms of calpain by E-64 and Ep-420*

The apparent second-order rate constants were determined as described in the Experimental section. 'Autolysed calpain' is calpain that has been incubated in the presence of 5mM-$CaCl_2$ for 40min at 22°C. 'Degraded calpain' is the material that is eluted from the Mono Q column after the first peak of calpain.

	Rate constant ($M^{-1} \cdot s^{-1}$)		
Inhibitor	Calpain	Autolysed calpain	Degraded calpain
E-64	7500	8050	8070
Ep-420	440	460	460

Autolysis of calpain

Calpain is susceptible to rapid autolysis in the presence of Ca^{2+} (see the introduction). In determining the kinetic constants in Table 1, no indication of multiple rates of inactivation was observed. However, during the course of the experiments (2–40min) autolysis will have occurred. Rate constants for inactivation were therefore obtained for calpain that had been preincubated at 22°C in 5mM-$CaCl_2$ for 40min ('autolysed calpain'). The results are shown in Table 2, together with data for 'degraded calpain'. 'Autolysed calpain' has a completely degraded 28000-M_r chain and a partially degraded 78000-M_r chain (see Fig. 2). 'Degraded calpain' has a partially degraded 28000-M_r chain and an intact 78000-M_r chain (see Fig. 1).

The time course of autolysis was investigated as follows. A mixture of calpain (about 4nmol) and [^{3}H]Ac-Ep-459-labelled calpain (about 3nmol) was incubated at 22°C in 2.5ml of 50mM-Tris/HCl buffer, pH7.5, containing 0.1M-NaCl, 5mM-$CaCl_2$ and 0.05% (v/v) 2-mercaptoethanol. At various times, samples were removed and either assayed for calpain activity or diluted into 20% (w/v) trichloroacetic acid for subsequent analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The results of such an experiment are shown in Fig. 2. The time courses for loss of enzymic activity and the changes in electrophoretic behaviour were the same for mixtures of calpain with labelled calpain as for calpain alone. If the samples were run in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis without reduction, the results were the same as those in Fig. 2(*a*). Similarly, the time course was not affected by varying the protein concentration in the range 0.1–1.0mg/ml. When labelled calpain was incubated in the absence of active enzyme, no degradation was observed.

Discussion

The rate constants for inactivation of calpain by peptide epoxides (Table 1) show, firstly, that the epoxide group is important for rapid inactivation; Ep-475 is a much better inactivator than DC-11. Similarly, the stereochemistry of the group is important, since E-64(L) inactivates much more rapidly than E-64(D). Comparison of the rates for Ep-459 and Ac-Ep-459 suggests that the positive charge on Ep-459 is not important. Increasing the length of the carbon chain of Ep-459 to give Ep-479 increases the rate of inactivation, but replacing the aliphatic chain with a benzyloxycarbonyl group, as in Ep-460, gives a much more dramatic increase in the rate of inactivation. This suggests that calpain may have a hydrophobic binding site that will accommodate this group.

Previous reports of the inhibition of calpain by epoxide derivatives have simply quoted the concentration of inhibitor required for 50% inhibition (ID_{50}) after incubation with calpain for a fixed time period, in the presence or in the absence of substrate (Sugita *et al.*, 1980; Suzuki, 1983; Hara & Takahashi, 1983). Measurement of ID_{50} values is not appropriate for irreversible inhibitors; however, the ID_{50} values for E-64(L), Ep-475 and Ep-459 quoted by Suzuki (1983) are consistent with the data in Table 1.

The inactivation of other cysteine proteinases by epoxide derivatives has been investigated by Barrett *et al.* (1982). A direct comparison of the rate constants with those for calpain is not possible, as the results were obtained at different temperatures. However, assuming a 2-fold change in rate for a 10°C change in temparature, calpain is inhibited by E-64 approx. 20-fold more slowly than papain and approx. 3-fold more slowly than cathepsin B. Comparison of the relative rates of inactivation for the various inhibitors shows that calpain resembles papain and cathepsins B and L in that the fastest rates of inactivation are observed with E-64, Ep-475, Ep-460 and Ep-479. However, each enzyme shows a unique spectrum of specificity for the various epoxide inactivators. In contrast with papain and the cathepsins, calpain was not inactivated rapidly by Z-Phe-Phe-CHN_2, Z-Phe-Ala-CHN_2 or Pro-Phe-Arg-CH_2Cl.

Calpain autolyses in the presence of Ca^{2+} (Fig. 2). The first step is the degradation of the 28000-M_r chain to a 17000-M_r fragment. This occurs within the first 5min of incubation. The susceptibility of the small subunit to rapid degradation has been noted previously (Mellgren *et al.*, 1982; Hathaway *et al.*, 1982; Dayton, 1982). Degradation of the 78000-M_r chain occurs more slowly, but is complete within 2h. Autolysis of calpain labelled with [^{3}H]Ac-Ep-459 shows that the 78000-M_r chain is

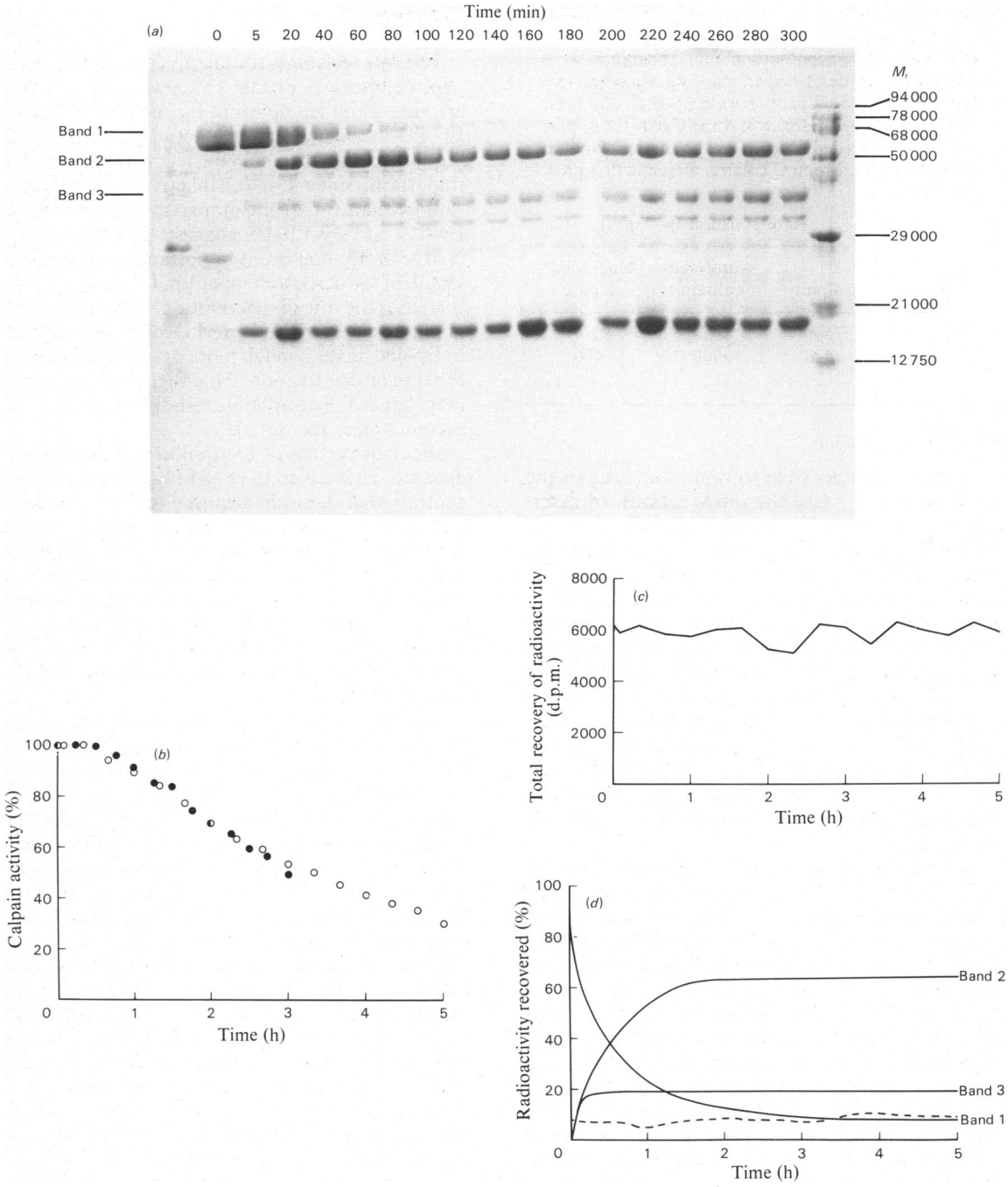

Fig. 2. *Autolysis of calpain*

A mixture of calpain and calpain labelled with [^{3}H]Ac-Ep-459 was incubated at 22°C in 50mM-Tris/HCl buffer, pH 7.5, containing 0.1M-NaCl, 5mM-$CaCl_2$ and 0.05% (v/v) 2-mercaptoethanol. At various times, samples were removed and either assayed for calpain activity or analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. (*a*) Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of calpain samples taken during autolysis. (*b*) Calpain activity during autolysis of either a mixture of calpain with [^{3}H]Ac-Ep-459-labelled calpain (○) or calpain alone (●). (*c*) Total recovery of radioactivity for each gel sample track shown in (*a*). (*d*) Radioactivity recovered in gel bands 1, 2 and 3 and the remainder of each sample track (----) expressed as a percentage of the total radioactivity in each sample track. The radioactivity was eluted from the gel and counted as described in the Experimental section.

first degraded to yield fragments of M_r 55000 (major) and 37000 (minor), but that the time course of formation of these does not show a precursor–product relationship, suggesting that there is more than one breakdown pathway. Unlabelled fragments of the 78000-M_r chain are presumably responsible for the bands at M_r 32000 and 28000. It seems likely that the band at M_r 17000 contains unlabelled material from the 78000-M_r chain, as well as the degraded 28000-M_r chain, since the band is heavily stained in the later part of the time course. The situation analysed in the autolysis experiments is complex, because the starting material is heterogeneous. The calpain at the beginning of the experiments comprises unlabelled material (78000-M_r and 28000-M_r chains) and labelled calpain (78000-M_r chain and degraded light chain). However, since the time courses of electrophoretic changes and loss of enzyme activity are similar for experiments with calpain or calpain plus labelled calpain, this heterogeneity seems not to affect the results.

Other workers have observed that the first step in the autolysis of the higher-M_r polypeptide results in only a small decrease in M_r, e.g. from 80000 to 76000 (Hathaway *et al.*, 1982).This is not seen in Fig. 2. The possibility that the calpain had already undergone this autolysis step during preparation seems to be ruled out by the fact that the enzyme required 1–3mM-Ca^{2+} for full activity, since it is reported that the initial cleavage is associated with a decrease to micro-molar Ca^{2+} requirement (Suzuki *et al.*, 1981*a*,*b*).

The proteolysis of calpain incubated in the presence of Ca^{2+} is essentially complete in 2h, but, at this stage, the enzyme retains approx. 70% of its original activity (Fig. 2*b*). It appears that, as reported by others (Suzuki *et al.*, 1981*a*,*b*; Hathaway *et al.*, 1982), the rate of loss of activity is much slower than the degradation of the 78000-M_r active-site-containing polypeptide. The fact that the reactivity of the active site is not affected by the degradation of the 78000-M_r peptide means that valid kinetic constants for the inactivation of calpain could be obtained despite autolysis, and that 'autolysed calpain' and 'degraded calpain' have the same kinetic constants as native calpain (Table 2). This confirms the data of Suzuki *et al.* (1981*a*), who found that calpain incubated with Ca^{2+} for 60min at 0°C had the same ID_{50} value for E-64 as native calpain.

Autolytic degradation must be an intermolecular process, since inactive labelled calpain is degraded. Autolysis has previously been suggested to be intermolecular (Suzuki *et al.*, 1981*a*) or intramolecular (Hathaway *et al.*, 1982; Mellgren *et al.*, 1982). The results in Fig. 2 suggest that, after the addition of Ca^{2+} to a solution of calpain, there is intermolecular proteolysis of the polypeptide chains via multiple pathways followed by slow loss of enzymic activity, which could be the result of an intramolecular process.

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