Inhibition of chicken calpain II by proteins of the cystatin superfamily and α_2 -macroglobulin

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Inhibition of chicken calpain II by proteins of the cystatin superfamily and α_2 -macroglobulin was investigated. Human liver cystatins A and B, human cystatin C, chicken cystatin and rat T-kininogen were found not to be inhibitory. Inhibition was, however, observed for bovine and rat kininogens, with K_i (inhibition constant) values of 0.8 nM and 30 nM respectively. α_2 -Macroglobulin inhibits calpain with an initial rate constant of the order of $3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$. Calpain complexed with α_2 -macroglobulin showed only limited reactivity towards azocasein, but reacted readily with the peptide substrate Suc-Leu-Tyr-4-methyl-7-coumarylamide and with L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-guanidino)butane (E-64). The calpain in the complexes was at least partially protected from loss of activity due to autolysis. The calpain- α_2 -macroglobulin complexes contained both the calpain subunits.

INTRODUCTION

Protein inhibitors of the papain-like cysteine proteinases include α_2 -macroglobulin and members of the cystatin superfamily. α_2 -Macroglobulin is a tetrameric plasma protein of M_r 725 000 that inhibits all classes of endopeptidase by a 'trap' mechanism (Barrett & Starkey, 1973). The cystatin superfamily comprises proteins that are homologous with chicken cystatin (Müller-Esterl *et al.*, 1985b; Barrett *et al.*, 1986a). These proteins have been divided into three families: family 1, members of which have M_r values of about 11 000 and no disulphide bonds; family 2, members of which have M_r values of about 13 000 and contain two disulphide bonds; and family 3 (kininogens), members of which have nine disulphide bonds. Two forms of kininogen, products of the same gene (Kitamura *et al.*, 1985; Takagaki *et al.*, 1985), are found in the plasma of most species; high- M_r kininogen (H-kininogen, M_r about 110 000) and low- M_r kininogen (L-kininogen; M_r about 60 000). These proteins are identical with α -cysteine proteinase inhibitor (Ohkubo *et al.*, 1984; Müller-Esterl *et al.*, 1985a; Sueyoshi *et al.*, 1985). The kininogens are single-chain glycoproteins which, when the kinin peptides are released after cleavage by kallikreins, are composed of an *N*-terminal heavy chain disulphide-bonded to the *C*-terminal light chain. The heavy chains of L-kininogen and H-kininogen are identical and are constructed from three cystatin-like segments. In rat, additional kininogens have been identified, termed 'T-kininogens', that are expressed in response to acute inflammation (Anderson & Heath, 1985; Furuto-Kato *et al.*, 1985; Kageyama *et al.*, 1985). Members of the cystatin superfamily inhibit papain-like cysteine proteinases, including the lysosomal enzymes cathepsins B, H and L, by forming tight enzyme-inhibitor complexes; however, the exact nature of the interaction is unknown (for review see Barrett *et al.*, 1986b).

Calpains are Ca^{2+} -activated cysteine proteinases that are widely distributed in the cytoplasm of animal cells (for review see Murachi, 1984). The enzymes are composed of two subunits; one of M_r 80 000 that has four domains, including a papain-like active-site domain, and a calmodulin-like Ca^{2+} -binding domain; and one of M_r 30 000 that has two domains, an *N*-terminal glycine-rich domain and a *C*-terminal calmodulin-like Ca^{2+} -binding domain (Ohno *et al.*, 1984; Sakihama *et al.*, 1985). Two types of calpains are currently defined: calpains I, which are fully active at micromolar Ca^{2+} concentrations, and calpains II, which require millimolar Ca^{2+} for full activity. The types have distinct 80 000- M_r subunits but identical 30 000- M_r subunits. In the presence of Ca^{2+} , calpains undergo autolysis with initial breakdown of the light chain and *N*-terminal cleavage of the heavy chain, followed by further breakdown of the heavy chain and loss of enzymic activity (Suzuki *et al.*, 1981; Hathaway *et al.*, 1982; Parkes *et al.*, 1985).

The active-site domain of calpains shows only 20–25% homology with papain, and the overall structure of calpains is very different from the plant and lysosomal cysteine proteinases. It is therefore of interest to consider whether the protein inhibitors of the papain-like cysteine proteinases are also effective inhibitors of calpains. Members of the cystatin superfamily have not been extensively investigated as inhibitors of calpains. However, there are some reports that the low- M_r inhibitors are not effective against calpains: rat cystatin β (family 1) did not inhibit rat skeletal-muscle calpain (Kominami *et al.*, 1982); human amniotic-fluid cystatin did not inhibit bovine lung calpain (Rohrlich *et al.*, 1985); and chicken cystatin C did not inhibit chicken gizzard calpain II (Johnson *et al.*, 1984). In contrast with these results, human H-kininogen inhibited human erythrocyte calpain I, human kidney calpain II, human platelet calpain and porcine skeletal-muscle calpain; and human L-kininogen inhibited chicken gizzard calpain II and porcine skeletal-

Abbreviations used: [S], total substrate concentration; [I], total inhibitor concentration; K_i , inhibition constant; $K_{i(\text{app})}$, apparent inhibition constant in the presence of substrate; v_0 , rate of reaction in the absence of inhibitor; v_i , rate of reaction at steady state in the presence of inhibitor; k_2 , second-order rate constant; 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; Z-, benzoyloxycarbonyl.

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muscle calpain (Sasaki *et al.*, 1983; Higashiyama *et al.*, 1986; Salvesen *et al.*, 1986; Schmaier *et al.*, 1986). In the case of human L-kininogen, the inhibition of calpain was shown to be due to the central cystatin-like segment of the heavy chain; the other cystatin-like segments were not inhibitory (Salvesen *et al.*, 1986).

In view of these diverse results, I decided to test a further range of family-1, family-2 and family-3 cystatins from various species, as inhibitors of chicken gizzard calpain II. α_2 -Macroglobulin has been reported to be an inhibitor of calpain, as well as inhibiting other cysteine proteinases (Sasaki *et al.*, 1983; Schmaier *et al.*, 1986), thus the reaction of α_2 -macroglobulin with calpain was also investigated. The results of these studies on the interaction of calpain with protein inhibitors of papain-like cysteine proteinases are presented.

EXPERIMENTAL

Materials

Papain, porcine pancreatic trypsin, di-isopropyl phosphorofluoridate and E-64 were 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 stock solution of 25 mM 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 chicken gizzard calpain II. Calpain II was prepared from chicken gizzard smooth muscle and the active-site concentration was determined by titration with E-64 as described previously (Parkes *et al.*, 1985).

Preparation of kininogens and cystatins. Kininogens were purified from bovine and rat plasmas by chromatography on carboxymethyl-papain-Sepharose in the presence of proteinase inhibitors, as described by Müller-Esterl *et al.* (1987). For each species the product was a mixture of H- and L-kininogens. Rat T-kininogen was generously given by Dr. F. Gauthier (Laboratoire de Biochimie, Faculté de Médecine, 2 bis, Bd. Tonnellé, 37032 Tours, France), and had been prepared as described by Esnard & Gauthier (1983).

Cystatins A and B (family 1) were prepared from human liver by the method of Green *et al.* (1984). Chicken cystatin (family 2) was prepared from egg white by the procedure of Anastasi *et al.* (1983). Human cystatin C (family 2) was kindly given by Dr. A. Grubb (Department of Clinical Chemistry, University of Lund, Malmö General Hospital, Malmö, Sweden), and had been purified from the urine of patients with kidney failure as described by Löfberg *et al.* (1981).

The concentration of inhibitor in solutions of purified family-1, family-2 and family-3 cystatins was found by titration with papain that had itself been titrated with E-64 (Anastasi *et al.*, 1983), assuming a 1:1 binding ratio for papain with the low- M_r inhibitors, with a 2:1 ratio for papain and kininogens (Salvesen *et al.*, 1986).

Preparation of α_2 -macroglobulin. α_2 -Macroglobulin was generously given by Dr. M. E. Davies of this laboratory and was purified from fresh human plasma by poly(ethylene glycol) precipitation, zinc chelate affinity chromatography and gel filtration with Ultrogel AcA-34

as described by Davies *et al.* (1987). Analysis on non-SDS/5%-(w/v)-polyacrylamide gels showed that the protein was all the electrophoretically slow form of α_2 -macroglobulin (Barrett *et al.*, 1979). The concentration of α_2 -macroglobulin solutions was found by assuming an $A_{280}^{1\%}$ of 9.1 (Dunn & Spiro, 1967).

Assay of calpain activity. For the assays with azocasein as a substrate, calpain was incubated in 0.5 ml of 100 mM-Tris/acetate buffer, pH 7.5, containing 100 mM-KCl, 5 mM- CaCl_2 and 0.02% (v/v) 2-mercaptoethanol at 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 μM -NH $_2$ Mec 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- CaCl_2 , 1 mM-dithiothreitol, 0.1% Triton X-100 and 250 μM -Suc-Leu-Tyr-NHMec as substrate. The assays were started by the addition of enzyme. The K_m determined for calpain and Suc-Leu-Tyr-NHMec was 4×10^{-4} M.

Quantification of the inhibitory activity of members of the cystatin superfamily. The K_i values for calpain and members of the cystatin superfamily were determined by use of continuous-rate fluorimetric assays. The assays, set up as described above, were started by the addition of enzyme (31 nM final concn.), and the inhibitor was added 3 min later. Data were obtained at different inhibitor concentrations, and K_i values were determined from plots of the form $[\text{I}]_i/(1-v_i/v_0)$ versus v_0/v_i , which have slope equal to $K_i [([S]_i + K_m)/K_m]$, assuming competitive inhibition (Henderson, 1972).

The K_i values for papain and members of the cystatin superfamily were determined similarly using Z-Phe-Arg-NHMec as substrate. The assays were done at 30 °C in 50 mM-sodium phosphate, pH 6.8, containing 1 mM-EDTA, 0.01% Brij-35, 1 mM-dithiothreitol and 10 μM -substrate, with a final enzyme concentration of 0.01 nM (Gounaris *et al.*, 1984).

Electrophoresis. SDS/polyacrylamide-gel electrophoresis was with gels of 10% (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.

RESULTS

Reaction of calpain with members of the cystatin superfamily

The K_i values for calpain and members of the cystatin superfamily were determined as described under 'Methods' and are given in Table 1. For the family-1 and -2 cystatins and rat T-kininogen, no inhibition of calpain was observed. For these inhibitors a K_i value of > 10000 nM is quoted. K_i values above 10000 nM could not be measured because there was an upper limit to the

Table 1. K_i values for calpain and papain with members of the cystatin superfamily

K_i values were determined by use of continuous rate fluorimetric assays and analysis by the method of Henderson (1972) as described under 'Methods'. Abbreviation used: nd, not determined.

Inhibitor	Family	K_i (nM)	
		Calpain	Papain
Human liver cystatin A	1	> 10000	0.019*
Human liver cystatin B	1	> 10000	0.120*
Human cystatin C	2	> 10000	< 0.005†
Chicken cystatin	2	> 10000	< 0.005*
Bovine kininogens	3	0.8	0.016
Rat kininogens	3	30.0	0.020
Rat T-kininogen	3	> 1000	nd
Human L-kininogen	3	1.0‡	0.015‡

* Data from Green *et al.* (1984).

† Data from Barrett *et al.* (1984).

‡ Data from Salvesen *et al.* (1986).

concentration of inhibitor that could be used and a limit to the duration of the assays (as the calpain activity decreased owing to autolysis in long-term experiments). The K_i value for papain and rat T-kininogen was not determined; however, the rat T-kininogen did inhibit papain, as it was possible to titrate the inhibitor with papain. Sueyoshi *et al.* (1985) reported a value of 0.19 nM for the K_i of papain and rat T-kininogen, and values for papain and bovine and rat kininogens of the same order of magnitude as those in Table 1.

Reaction of calpain with α_2 -macroglobulin

Incubation of calpain with an excess of α_2 -macroglobulin at 20 °C in 50 mM-Tris/HCl, pH 7.5, containing 5 mM- CaCl_2 for 15 min, followed by assaying for calpain activity with azocasein as a substrate, shows that α_2 -macroglobulin does inhibit chicken calpain II. At high concentrations of α_2 -macroglobulin the calpain activity did not fall below 18% (S.E.M. ± 4 , $n = 5$) of the activity in the absence of α_2 -macroglobulin. This suggests that azocasein has restricted access to the active site of calpain when the enzyme is complexed with α_2 -macroglobulin. Other α_2 -macroglobulin-proteinase complexes have been shown to have limited reactivity with high- M_r substrates (Barrett & Starkey, 1973).

The ability of calpain complexed with α_2 -macroglobulin to react with the peptide substrate Suc-Leu-Tyr-NHMec was also investigated. Calpain (150 pmol) was incubated at 20 °C in 0.4 ml of 50 mM-Tris/HCl, pH 7.5, containing 10 mM- CaCl_2 in the presence (710 pmol) or absence of α_2 -macroglobulin. After 0, 10 or 45 min the mixture was diluted with the components required for the fluorogenic assay (see under 'Methods'), and the enzymic activity against Suc-Leu-Tyr-NHMec determined (Table 2). When complexed with α_2 -macroglobulin, calpain was still substantially active against the peptide substrate. Incubation of calpain with Ca^{2+} normally results in loss of activity, owing to autolysis,

Table 2. Reaction of calpain- α_2 -macroglobulin complexes with Suc-Leu-Tyr-NHMec

Calpain was preincubated with Ca^{2+} (10 mM) in the absence or presence of α_2 -macroglobulin. The enzyme activity against Suc-Leu-Tyr-NHMec was determined at various times. For details, see the text.

Period of preincubation (min)	Activity (%)	
	Without α_2 -macroglobulin	With α_2 -macroglobulin
0	100	—
10	86	97
45	45	70

but the calpain in the complexes was at least partially protected from this loss of activity. Addition of E-64 (8 μM final concn.) to the fluorogenic assays of calpain- α_2 -macroglobulin complexes resulted in complete inhibition of enzymic activity. Thus the active site of calpain in the complexes is accessible to the peptide epoxide.

The rate of reaction of calpain with α_2 -macroglobulin was determined by incubating equimolar amounts of enzyme and inhibitor in 50 mM-Tris/HCl, pH 7.5, containing 5 mM- CaCl_2 at 20 °C, and assaying samples taken at various times for enzymic activity using azocasein as a substrate. The second-order rate constant for inactivation was found from the slope of a plot of the reciprocal of the enzyme concentration versus time; $k_2 = 29 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ (S.E.M. = 15×10^3 , $n = 8$). The second-order rate constant could not be determined very accurately for the following reasons: (1) calpain- α_2 -macroglobulin complexes show some reactivity towards azocasein; (2) even though the dilution of the enzyme-inhibitor mixture into the assay system was at least 10-fold, reaction between enzyme and inhibitor continues during the relatively long assay time (30 min); and (3) the analysis assumes equimolar concentrations of enzyme and inhibitor, but these values are subject to error.

Composition of calpain- α_2 -macroglobulin complexes

As calpain is one of the larger proteinases to be inhibited by α_2 -macroglobulin, I decided to determine whether the complete 110000- M_r heterodimer, or only a small part of the enzyme, was trapped by the inhibitor. Thus the composition of the calpain- α_2 -macroglobulin complexes was investigated as follows. Calpain (about 7.5 nmol) and α_2 -macroglobulin (about 6.5 nmol) were incubated in 50 mM-Tris/HCl, pH 7.5, containing 10 mM- CaCl_2 for 15 min at 20 °C. A sample of the mixture was assayed with azocasein; 35% of the caseinolytic activity of calpain remained, corresponding to approx. 80% inhibition. [^3H]Ac-Ep-459 (135 nmol) was then added and the incubation continued for 30 min. The mixture was again assayed for enzyme activity with azocasein; no enzymic activity was detected. The mixture was made 25 mM in EDTA and chromatographed on a column (90 cm $\times$ 1.5 cm diam.) of Ultrogel AcA-34 in 50 mM-Tris/HCl, pH 7.5, containing 5 mM-EDTA. The elution profile of the column is shown in Fig. 1. The presence of radioactivity in the high- M_r calpain- α_2 -macroglobulin peak shows that the complex contained the large calpain subunit. Fractions from the column

Fig. 1. Chromatography of calpain- α_2 -macroglobulin complexes on Ultrogel AcA-34

Calpain- α_2 -macroglobulin complexes were prepared and labelled with [3 H]Ac-Ep-459 as described in the text. The complexes were chromatographed on a column (90 cm $\times$ 1.5 cm diam.) of Ultrogel AcA-34 in 50 mM-Tris/HCl, pH 7.5, containing 5 mM-EDTA. The protein concentration (A_{280} , —) and radioactivity (d.p.m. in 50 μ l aliquots, ----) of the fractions were determined.

were subjected to SDS/10%-(w/v)-polyacrylamide-gel electrophoresis (Fig. 2). α_2 -Macroglobulin runs as a single band of M_r 185000 on this gel (track 4), corresponding to the quarter subunit. The calpain has a band at M_r 80000 corresponding to the heavy chain, and bands at M_r 28000, 25600 and 23600 corresponding to the light chain and degraded forms of the light chain (track 3). The calpain which was not complexed with α_2 -macroglobulin (elution volume 88 ml) is partially autolysed, having bands at M_r 80000 and 18000 (track 2). The calpain- α_2 -macroglobulin complexes (elution volume 58 ml) contain a band at M_r 18000, a band at M_r 98000–85000, a band at M_r 185000 and a series of bands of M_r greater than 240000 (track 1). The α_2 -macroglobulin has been cleaved in the 'bait' region to give the fragments of M_r 95000 and 85000 (not resolved on the gel). Comparison of tracks 1 and 2 shows that the complexes contain the autolysed light subunit of calpain (M_r 18000). The sample in track 1 was overloaded so as to demonstrate clearly the presence of the 18000- M_r band from the calpain small subunit. A similar overloaded track of trypsin- α_2 -macroglobulin complexes (track 5) shows no band at 18000 M_r . This is additional evidence that the 18000- M_r band in track 1 is derived from calpain.

DISCUSSION

The results presented in Table 1 show that none of the low- M_r cystatins tested (families 1 and 2) inhibit calpain. These observations are consistent with previous reports of the lack of inhibition of calpain by rat cystatin β , chicken cystatin C and amniotic-fluid cystatin (Komin-

Fig. 2. Analysis of calpain- α_2 -macroglobulin complexes by SDS/10%-(w/v)-polyacrylamide-gel electrophoresis

Track 1, calpain- α_2 -macroglobulin complexes eluted at 58 ml from the Ultrogel AcA-34 column (Fig. 1); track 2, calpain not incorporated into complexes, eluted at 88 ml from the Ultrogel AcA-34 column (Fig. 1); track 3, calpain; track 4, α_2 -macroglobulin; track 5, trypsin- α_2 -macroglobulin complexes prepared by allowing α_2 -macroglobulin to react, in 50 mM-Tris/HCl, pH 7.5, containing 5 mM-EDTA, with a 4-fold molar excess of trypsin, followed after 1 min at room temperature by a 10^3 -fold excess of di-isopropyl phosphorofluoridate.

ami *et al.*, 1982; Johnson *et al.*, 1984; Rohrich *et al.*, 1985). Inhibition, with K_i values in the nanomolar range, was, however, found for the interaction of chicken calpain II with bovine and rat kininogens and for human L-kininogen, as reported previously (Salvesen *et al.*, 1986). Surprisingly the rat T-kininogen was not an effective inhibitor of calpain, although it does inhibit papain and cathepsins B, H and L (Sueyoshi *et al.*, 1985; Gauthier *et al.*, 1987).

All of the inhibitors discussed are members of the cystatin superfamily. However, their amino acid sequences are not all closely related; the superfamily is divided into three distinct families. Family-1 and family-2 cystatins show respectively 80–90% and 72–83% differences from the kininogens (Barrett *et al.*, 1986b). Thus a difference in inhibition characteristics between kininogens and family-1 and -2 cystatins is not unexpected. Within the kininogens (family-3 cystatins) there is stronger sequence homology. The central cystatin-like domain of rat T-kininogen 1 shows 44% difference from the same domain of human and bovine kininogens, and rat T-kininogen 1 shows 19% differences from rat L-kininogen (Furuto-Kato *et al.*, 1985). Nevertheless the

results in Table 1 show that these proteins have functional differences.

Why certain cystatin-like domains inhibit calpain, whereas others do not, remains elusive. The results presented here show that chicken calpain II is inhibited by kininogens from a range of species, but that not all kininogens are inhibitory, as evidenced by the results with rat T-kininogen.

α_2 -Macroglobulin is an effective inhibitor of calpain. The rate constant for the reaction is of the order of $3 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$. This is intermediate in the range of values that have been found for the reaction of proteinases with α_2 -macroglobulin. For example the rate constant determined for bovine trypsin is $1.5 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ and that for thrombin is $5 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Travis & Salvesen, 1983). The calpain complexed with α_2 -macroglobulin shows only limited reactivity (18%) with the protein substrate azocasein, presumably because the substrate cannot easily gain access to the active site of the enzyme. By contrast, E-64 and the peptide substrate Suc-Leu-Tyr-NHMec react readily with calpain complexed to α_2 -macroglobulin. The calpain in the complexes is at least partially protected from loss of activity due to autolysis (Table 2). This observation is consistent with the fact that the later autolytic reactions are intermolecular (Parkes *et al.*, 1985) and the idea that the loss of activity associated with autolysis is due to dissociation of the autolytic fragments after autolysis is complete [the preceding paper (Crawford *et al.*, 1987)].

The results in Figs. 1 and 2 demonstrate the presence of the active site from the heavy chain and a major fragment (M_r 18000) of the light chain of calpain in the calpain- α_2 -macroglobulin complexes. This is consistent with the observed rate of reaction of calpain with α_2 -macroglobulin, which is high in comparison with the rates of extensive autolytic breakdown of the heavy chain of calpain and dissociation of the calpain fragments (Parkes *et al.*, 1985; Crawford *et al.*, 1987). The presence of the 18000- M_r fragments rather than the intact 30000- M_r subunit in the complexes does, however, suggest that the initial events in autolysis happen more quickly than inhibition or that intramolecular autolysis continues after complex-formation. Thus α_2 -macroglobulin traps a 98000–110000- M_r form of the enzyme. α_2 -Macroglobulin is capable of trapping other large proteinases, such as plasmin ($M_r \sim 77000$) and kallikrein ($M_r \sim 88000$), but calpain is the largest proteinase so far found to be inhibited by α_2 -macroglobulin. In view of these results, models for the interaction of α_2 -macroglobulin with proteinases (e.g. Feldman *et al.*, 1985) need to take into account that there must be sufficient space inside the trap to accommodate the 98000–110000 M_r form of calpain.

Members of cystatin families 1 and 2 occur intracellularly, where calpains are found, but since these inhibitors are not effective against calpains, they cannot contribute to the control of calpain activity inside cells. α_2 -Macroglobulin and some of the kininogens, which are inhibitors of calpains, are found extracellularly. Thus they can only have a physiological role in controlling calpain activity if the calpains are released from cells.

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