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# Structural modifications associated with the change in $Ca^{2+}$ sensitivity on activation of m-calpain

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Autolysis of the $Ca^{2+}$-dependent cysteine protease m-calpain involves cleavage of the large (80 kDa) and small (30 kDa) subunits of the enzyme, and an increase in $Ca^{2+}$ sensitivity. The appearance of increased $Ca^{2+}$ sensitivity was found to correlate with the cleavage of the large subunit after residue 9.

$Ca^{2+}$-dependent protease; $Ca^{2+}$ sensitivity; Calpain activation; Autolysis

## 1. INTRODUCTION

Calpains are $Ca^{2+}$ dependent cysteine proteinases found in the cytosol of animal cells. They have been implicated in a wide variety of cellular events initiated by $Ca^{2+}$ signals (for review see [1]). Two forms of calpain are well characterised. M-calpain, active in vitro at millimolar $Ca^{2+}$ concentrations, and $\mu$-calpain, active in vitro at micromolecular $Ca^{2+}$ concentrations. Both forms have two subunits of 80 kDa and 30 kDA; the amino acid sequence of the 80 kDa subunit differs in the two forms. On binding $Ca^{2+}$ to the calmodulin-like domains at the C-termini of the subunits, calpains become active and begin to self-digest (autolyse). One consequence of autolysis is a change in the $Ca^{2+}$ sensitivity of calpains; the enzymes become active at lower $Ca^{2+}$ concentrations [2,3]. The change in $Ca^{2+}$ sensitivity has been proposed to play a role in enzyme activation in vivo [4].

The initial autolytic events involve proteolytic cleavages in both calpain subunits. Many authors report conversion of the 30 kDa small subunit to an approximately 18 kDa polypeptide for both m- and $\mu$-calpain (e.g. [3,5–7]). Autolysis of the large subunit of $\mu$-calpain initially involves degradation at the N-terminus and formation of polypeptides of approximately 78 kDa and 76 kDa [8,9] or only 76 kDa [5]. These polypeptides can readily be resolved from the 80 kDa polypeptide on SDS-polyacrylamide gel electrophoresis. Autolysis of the large subunit of m-calpain also involves cleavage at the N-terminus. Imajoh et al. reported that the N-terminus of rabbit m-calpain was blocked and that autolysed m-calpain had an amino acid sequence corresponding to residue 20 onwards of the human m-calpain sequence [6,10]. For chicken m-calpain conversion from the 80 kDa polypeptide to a 76 kDa polypeptide can be observed on SDS-PAGE [3,11]; but for mammalian m-calpains no change in electrophoretic mobility is detectable [5,12,13]. For both forms of calpain these initial autolytic events are followed by the conversion of the large subunit to a polypeptide of approximately 50 kDa [7,11], and by a slow loss of enzyme activity.

There is some controversy concerning which autolytic event is responsible for the observed change in $Ca^{2+}$ sensitivity. DeMartino et al. [14] showed that an increase in $Ca^{2+}$ sensitivity occurred on formation of the 80 kDa plus 18 kDa heterodimeric form of m-calpain, suggesting that cleavage of the small subunit was responsible. In contrast, Suzuki et al. [11] and Imajoh et al. [6] proposed that the change in $Ca^{2+}$ sensitivity was associated with autolysis of the large subunit of m-calpain. We have analysed the autolysis of pig m-calpain and have correlated the change in $Ca^{2+}$ sensitivity with a novel cleavage in the large subunit of the protein.

## 2. EXPERIMENTAL

### 2.1. *Purification of m-calpain*

M-calpain was purified from pig kidney as described by Crawford et al. [15]. Enzyme activity was determined using azocasein as a substrate as described by Crawford et al. [16].

### 2.2. *Gel electrophoresis*

SDS-polyacrylamide gel electrophoresis was with gels of 12.5% (w/v) polyacrylamide and the buffer system described by Laemmli [17]. The samples were prepared by adding an equal volume of 125 mM Tris-HCl pH 6.8, containing 10 mM EDTA, 20% (v/v) glycerol, 10% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 5% (v/v) saturated Bro-

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mophenol blue, and incubating in a boiling water bath for 5 min. The gels were stained with Coomassie brilliant blue.

2.3. *Amino acid sequence analysis*

Amino acid sequence analysis was performed as described previously [15]. Briefly, the samples were run on SDS-PAGE, blotted onto 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.

2.4. *Assay of calpain $Ca^{2+}$ sensitivity*

Four incubation mixtures were set up at room temperature each containing m-calpain (50 $\mu$g) in 60 $\mu$l 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 2.5 mM EDTA, 4.5 mM $Ca^{2+}$ and 0.1% $NaN_3$. After 40 s, 2 min, 5 min and 10 min, respectively, five 10-$\mu$l samples were taken from each incubation mixture and the calpain activity determined using the azocasein assay with final $Ca^{2+}$ concentrations of 0.1 mM, 0.2 mM, 0.5 mM, 1.0 mM and 2.0 mM, respectively.

## 3. RESULTS

A time course of autolysis of pig m-calpain analysed by SDS-PAGE is shown in Fig. 1. Very rapid degradation of the 30 kDa subunit to the 18 kDa form is observed, followed by a slower loss of the 80 kDa subunit and appearance after about 15 min of peptides of 43 kDa, 24 kDa and 23 kDa. A different pattern is observed with pig $\mu$-calpain. In this case the two subunits are degraded simultaneously; 78 kDa and 76 kDa large subunit fragments can be detected on the gel (data not shown). Amino acid sequence analysis was performed on the large subunit of m-calpain autolysed for 0 min, 0.5 min, 5 min and 15 min (prepared using identical conditions to those used in the $Ca^{2+}$ sensitivity experiments and stopping the reaction with SDS gel sample buffer containing EDTA), and on the 43 kDa polypeptide at 120 min. The results are given in Table I and show that two successive cleavages occur at the N-terminus of m-calpain during the first 15 min of autolysis.

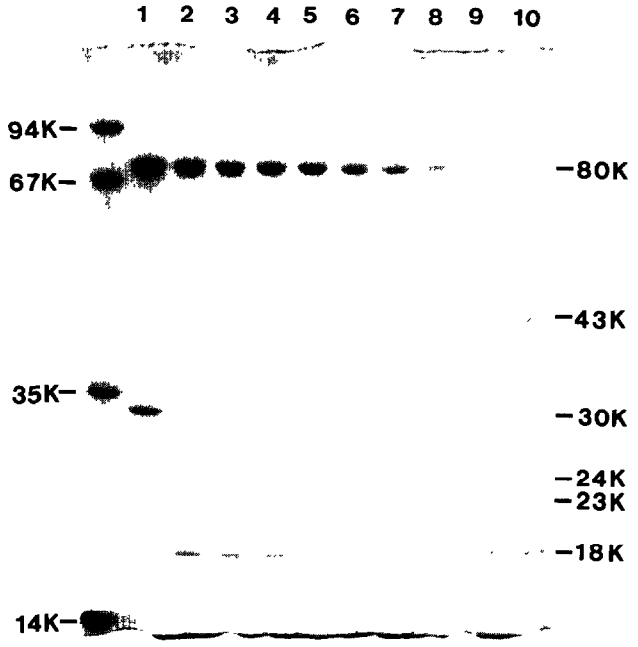


Fig. 1. Time course of autolysis of m-calpain analysed by SDS-PAGE. Calpain (60 $\mu$g) was incubated in 50 mM Tris-HCl pH 7.5 containing 100 mM NaCl, 5 mM EDTA, 0.01% monothioglycerol, 0.1% $NaN_3$ with 5 mM free $Ca^{2+}$ at room temperature. At various times samples (2 $\mu$g) were removed and run on 12.5% SDS-PAGE. The times were: 1, 0 s; 2, 5 s; 3, 15 s; 4, 30 s; 5, 1 min; 6, 5 min; 7, 15 min; 8, 30 min; 9, 1 h; 10,2 h.

The change in $Ca^{2+}$ sensitivity of m-calpain during autolysis was investigated as described in the experi-

Table I

Sequence analysis of autolysed calpain

| | |
|---|---|
| m-Calpain 80kDa Human [10] | MAGIAAKLAKDREAAEGLGSHERAIKYLNQDYEA |
| Sequence at 30 s | Blocked N-terminus (as 80kDa) |
| Sequence at 5 min | KDREAAEGLG |
| Sequence at 15 min | (~40%) KDREAAEGLG |
| | (~60%) SHERAVKYLN |
| Sequence at 2 h | SHERAVKYLN |
| Autolysed m-calpain Rabbit [10] | SXETAFKYLNQ |
| Autolysed m-calpain Chicken [21] | AEGVGEHNNAVKYLNQDYEA |
| $\mu$-Calpain 80kDa Human [20] | MSEEIITPVYCTGVSAQVQKQRARELGLGRHENAIKYLGQDYEQ |
| Autolysed $\mu$-calpain 78 kDa Bovine [8] | AQVQKTRAKELG |
| Autolysed $\mu$-calpain 76 kDa Bovine [8] | LGRHQNAIKYLGQDEQL |
| Autolysed $\mu$-calpain 78 kDa Bovine [19] | LGRHENAIKY |

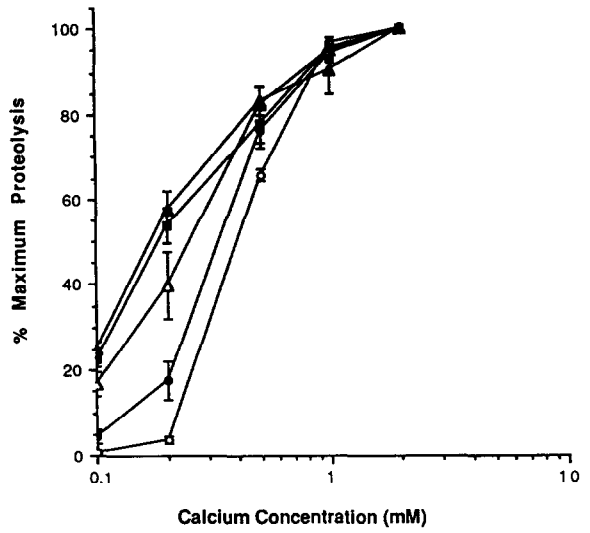


Fig. 2. $Ca^{2+}$-sensitivity of m-calpain during autolysis. The $Ca^{2+}$-sensitivity of m-calpain at various times during autolysis was assayed as described in the experimental section 2.4. Un-autolysed calpain —○—. 40sec autolysis —●—. 2min autolysis —△—. 5min autolysis —■—. 10min autolysis —▲—. The error bars represent 2 × S.E.M., $n = 4$.

mental section. The results are shown in Fig. 2. The $Ca^{2+}$ concentration required for 50% activity decreases from 0.40 mM to 0.18 mM after 5 min of autolysis, with no further significant change after 10 min. Reference to Table I suggests that the change in $Ca^{2+}$ sensitivity correlates with the first N-terminal cleavage in the large subunit; both events occur between 0.5 and 5.0 min of autolysis. After 40 s of autolysis, when the 80 kDa plus 30 kDa form of calpain is fully converted to the 80 kDa plus 18 kDa form, only a fraction of the total observable change in $Ca^{2+}$ sensitivity is apparent. It therefore seems unlikely that cleavage of the small subunit results in the change in $Ca^{2+}$ sensitivity. The second cleavage in the large subunit is incomplete at 15 min, whereas the change in $Ca^{2+}$ sensitivity is complete at 10 min. It therefore seems unlikely that the second cleavage of the large subunit results in the change in $Ca^{2+}$ sensitivity.

The heightened $Ca^{2+}$ sensitivity form of calpain has been described as unstable by Suzuki et al. [2,11]. The heightened $Ca^{2+}$ sensitivity form of m-calpain obtained as above, after 10 m autolysis, lost activity on dialysis against 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM EDTA, 0.1% 2-mercaptoethanol, 0.1% $NaN_3$. In contrast, Dayton [5] was able to retain activity of the autolysed forms of calpain on dialysis against 1 mM $KHCO_3$, 5 mM 2-mercaptoethanol, 0.5 mM EDTA. These observations suggest that the stability of the heightened $Ca^{2+}$ sensitivity forms may depend on the ionic strength of the buffer. Pig m-calpain was therefore autolysed for 5 min, dialysed against buffers with a range of ionic strengths and then assayed for activity. The results are shown in Table II and demonstrate that the heightened $Ca^{2+}$ sensitivity form of m-calpain is more stable at lower ionic strength.

Table II

Stability of 5-min autolysed m-calpain

| Buffer used for dialysis | Activity (%) |
|---|---|
| 50 mM Tris-HCl pH 7.5, 100 mM NaCl | 52 |
| 25 mM Tris-HCl pH 7.5, 50 mM NaCl | 78 |
| 12.5 mM Tris-HCl pH 7.5, 25 mM NaCl | 89 |

The dialysis buffers also contained 5 mM EDTA, 0.1% $NaN_3$ and 0.01% monothioglycerol. Activity of autolysed material is expressed as the % of the activity of an un-autolysed dialysed control; the data are the mean of two experiments.

## 4. DISCUSSION

The data presented in Fig. 2 and Table 1 indicate a correlation between generation of the heightened $Ca^{2+}$ sensitivity form of m-calpain and formation of the enzyme species with the large subunit cleaved after residue 9 and a 18 kDa small subunit. On SDS gels (Fig. 1) this species has a large subunit that appears identical to unautolysed calpain (80 kDa) and a 18 kDa small subunit. The first cleavage of the large subunit shown in Table 1 has not previously been reported. Imajoh et al. reported that autolysed m-calpain had an amino acid sequence corresponding to residue 20 onwards of the human m-calpain sequence [6,10]. This corresponds to the second cleavage in the large subunit reported here. Identification of two steps in the autolysis of the m-calpain large subunit makes the process more analogous to that seen with $\mu$-calpain. Zimmerman and Schlaepfer [8] identified two cleavage sites in $\mu$-calpain as shown in Table I. However in contrast to m-calpain, Zimmerman and Schlaepfer reported that the change in $Ca^{2+}$ sensitivity of $\mu$-calpain occurred after the second cleavage in the large subunit.

The results presented here are in agreement with Suzuki et al. [11] and Imajoh et al. [6] in demonstrating that the change in $Ca^{2+}$ sensitivity of m-calpain is associated with cleavage of the large subunit. Imajoh et al used reconstitution of subunits derived from either autolysed or unautolysed calpain to arrive at their conclusions. DeMartino et al. [14] suggested that cleavage of the small subunit was responsible for the change in $Ca^{2+}$ sensitivity of the enzyme, based on rates of subunit cleavage observed on SDS gels. It seems likely that they were misled by the difficulty of detection of m-calpain large subunit N-terminal cleavages on SDS gels. The lack of stability of the heightened $Ca^{2+}$ sensitivity form of m-calpain presumably contributes to the slow loss of enzyme activity that is observed following activation by $Ca^{2+}$.

There has recently been some interest in trying to observe the autolytic activation of calpain in vivo using antibodies that distinguish between the pre- and post-

autolysed forms of the enzyme. Croall et al. [18] have raised antibodies specific for unautolysed m- and $\mu$-calpain. Saido et al. [19] have raised antibodies against unautolysed and autolysed forms of $\mu$-calpain. The data in this paper should facilitate experiments using the antibody approach to investigate in vivo activation of m-calpain.

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