Autolysis parallels activation of μ-calpain

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The kinetics of autolysis and activation of μ-calpain were measured with microtubule-associated protein 2 (MAP2) as a very sensitive substrate. The initial rate of MAP2 hydrolysis was found to be a linear function of the autolysed 76 kDa form of μ-calpain large subunit at both 10 and 300 μM Ca^{2+}, and both straight lines intersected the origin. This finding supports the view that native μ-calpain is an inactive proenzyme and that activation is accompanied by autolysis. The first-order rate constant of autolysis, k_I(aut), was determined at different Ca^{2+} concentrations: the half-maximal value was at pCa^{2+} = 3.7 (197 μM Ca^{2+}), whereas the maximal value was 1.52 s^{-1}, at 30 °C. The Ca^{2+}-induced activation process was then monitored by using our novel, continuous fluorimetric assay with labelled MAP2 as substrate. The first-order rate constant of activation,

k_I(act), was derived as the reciprocal of the lag phase ('transit time') at the initial part of the progress curve: half-maximum was at pCa^{2+} = 3.8 (158 μM Ca^{2+}) and the maximum value was 2.15 s^{-1}. The good agreement between the kinetic parameters of μ-calpain autolysis and activation is remarkable. We claim that this is the first kinetically correct determination of the rate constant of autolysis of μ-calpain. Pre-activated μ-calpain has a Ca^{2+} requirement that is almost three orders of magnitude smaller [half-maximal activation at pCa^{2+} = 6.22 (0.6 μM Ca^{2+})]. We cannot exclude the possibility that the activation process involves other mechanistic steps, e.g. the rapid dissociation of the μ-calpain heterodimer, but we state that in our conditions *in vitro* autolysis and activation run in close parallel.

INTRODUCTION

Calpain (EC 3.4.22.17) is a Ca^{2+}-dependent cytoplasmic cysteine protease, the two ubiquitous forms of which have been thoroughly studied [1,2]. It is widely held that both forms (μ- and m-calpain) are inactive heterodimers undergoing autolytic activation in response to Ca^{2+} [3,4]. Recently this notion has been challenged by Suzuki and coworkers [5,6] who put forward the idea that the activation of calpain corresponds to subunit dissociation. It is not clear, however, whether dissociation is a consequence of autolysis or is the primary event in activation. Furthermore the dissociation model does not answer the intriguing question of the high, non-physiological Ca^{2+} requirement for the activation of calpain.

The controversy about calpain activation partly stems from the fact that measurements of activity, subunit dissociation, Ca^{2+}-sensitivity or autolysis have been based on enzyme and/or substrate conversions measured over lengthy (5–60 min) incubation periods. As these observations are far from the kinetically sound initial-rate conditions, the parameters in question vary with incubation time. In most cases the degree of autolysis has not been monitored at all, although μ-calpain undergoes rapid autolysis at high Ca^{2+} concentrations [7] and its autolysis cannot be excluded even at low Ca^{2+} concentrations [8,9]. Consequently most data on the Ca^{2+} sensitivity of 'native μ-calpain activity' are kinetically meaningless.

The idea that calpain is an inactive proenzyme [2,10] is generally supported by two lines of evidence. One is the appearance of a lag phase in the progress curve of substrate hydrolysis that, however, (1) has only been observed with m-calpain in the presence of Ca^{2+} [11,12], (2) seems to be too fast with μ-calpain and was only seen in the presence of Ba^{2+} [7], and (3) even with m-calpain it was observed only at low Ca^{2+} concentrations, leaving room for the criticism [13] that it might simply reflect sensitization to Ca^{2+} rather than activation of the enzyme. The other evidence would be that autolysis of μ-calpain large subunit

precedes substrate hydrolysis [7,14]. However, temporal order between percentage conversions varies with enzyme and substrate concentrations, which renders this argument irrelevant. We demonstrate this point below by showing that proteolysis of a sensitive substrate (MAP2) at low concentrations precedes autolysis of the enzyme.

The support for native calpain as an active enzyme [15] is also dubious on similar grounds. The argument that autolysis requires a higher Ca^{2+} concentration than activity does [16], for example, does not hold for mammalian vascular smooth-muscle calpain [17], and probably results from the application of a lengthy assay allowing significant autolysis and consequent sensitization to occur. Similarly, the observation that unautolysed m-calpain can react with an activity-dependent inhibitor such as *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) [13] may not hold for μ-calpain. Furthermore this result infers accessibility of the active centre of the enzyme for a small inhibitor molecule only and does not imply activity in the native state towards a bulky protein substrate.

All in all it seems that neither of these conflicting views has a firm experimental basis, which calls for further studies. Surprisingly, not even the Ca^{2+}-sensitivity of native μ-calpain has been determined under kinetically well defined conditions, which might explain why the respective values are scattered over such a wide range (2–75 μM [4] with occasional extremes such as 0.06 μM for activity [18] or 202 μM for autolysis [16]). It seems that most enzymological work devoted to μ-calpain suffers from kinetically improper practices and quantitative data should therefore be treated with caution.

A great many inconsistencies stem from the general use of caseinolytic assays, which are unsuitable for the temporal resolution of the early μ-calpain activation events that occur on the timescale of seconds. Our aim with the present work was to overcome this limitation by introducing MAP2 as substrate, which is far superior to casein [19] and permits activation measurements under initial-rate conditions. We use this system

to determine the true Ca^{2+}-sensitivity of μ-calpain autolysis and to relate activation to autolysis within a time frame experimentally not accessible so far.

EXPERIMENTAL

Materials

Erythrocyte concentrate was purchased from the National Institute of Haematology and Blood Transfusion (Budapest). Casein (prepared by the method of Hammarsten) was from Merck, DEAE-cellulose was from Whatman Ltd., phenyl-Sepharose, Q-Sepharose, Blue-Sepharose and Sephadex were from Pharmacia. All other chemicals used were analytical-purity preparations from Sigma Chemical Co. 5-([4,6-Dichlorotriazin-2-yl]amino)-fluorescein (DTAF) contained 10% dye adsorbed on Celite support. All buffers were prepared with ion-exchanged distilled water.

Preparation of proteins

Human erythrocyte μ-calpain was purified by the procedure described in [19], replacing the buffer with 10 mM Hepes, pH 7.5, containing 1 mM EGTA, 1 mM dithioerythritol, 0.1 mM PMSF and 1 mM benzamidine (calpain buffer). The purity of the preparation was at least 95% by SDS/PAGE and it had only Ca^{2+}-dependent proteolytic activity. Enzyme activity was routinely assayed by the method of Pintér and Friedrich [20]. MAP2 was purified as described in [19].

Determination of free Ca^{2+} concentration

The free Ca^{2+} concentration of an incubation mixture was measured with fura 2 below 2 μM [21] or by a commercial Ca^{2+} electrode above 50 μM. In the range not covered by either method, free Ca^{2+} concentration was calculated as described in [22].

Labelling of MAP2 with DTAF

DTAF–MAP2 was prepared as described in [19] with a slight modification: the Sephadex G-25 column was equilibrated with a buffer of 20 mM Hepes, pH 7.4, containing 0.5 mM dithioerythritol, 50 mM NaCl, 0.1 mM PMSF and 1 mM benzamidine.

Calpain activity assays

MAP2 assay

MAP2 (0.8 μM) was added to 0.35 μM μ-calpain preincubated for various times at either 10 or 300 μM free Ca^{2+} concentration in the calpain buffer at 0 °C. Several aliquots were quickly withdrawn only a few seconds apart, during which the progress of autolysis was negligible; the reaction was stopped by SDS sample buffer containing 50 mM EGTA, and subjected to SDS/PAGE. MAP2 band intensities were measured by densitometry and the initial rate of MAP2 hydrolysis was determined by fitting a linear function to the initial part of the progress curve.

Caseinolytic assay

The sensitivity of 'native μ-calpain activity' to Ca^{2+} was determined as described in [23]. This assay is based on measuring the liberation of trichloroacetic acid-soluble peptides from casein and includes a 10 min incubation period at 30 °C. Autolysed μ-calpain, i.e. the 76 kDa form prepared by a 5 min preincubation at 30 °C in the presence of 10 μM Ca^{2+}, was assayed in the same

way. These conditions were found by SDS/PAGE to yield pure 76 kDa μ-calpain virtually free of the native enzyme.

Fluorescence assay

Measurements were made on a JASCO FP 777 spectrofluorimeter at excitation and emission wavelengths of 490 and 520 nm respectively, in a 3 mm × 3 mm quartz cuvette, which allowed measurements in a 50 μl sample volume [19]. The response time was set to the minimum, 0.1 s. The increase in fluorescence intensity was recorded in the following standard assay of 50 μl at 30 °C: 20 mM Hepes, pH 7.4, 0.1 mM EGTA, 0.5 mM dithioerythritol, 50 mM NaCl, 0.2 mM PMSF, 1 mM benzamidine, 0.2 μM DTAF–MAP2, 0.02 μM μ-calpain, and $CaCl_2$ to ensure free Ca^{2+} concentrations from 0.6 μM to 3.2 mM. Transit times characteristic of the activation of the enzyme were determined by extrapolating the straight line fitted to the linear portion of the progress curves to zero substrate conversion [24].

Autolysis of μ-calpain

Autolysis of μ-calpain was followed at 30 °C in calpain buffer at various free Ca^{2+} concentrations by applying fast sampling. Autolysis in separate mixtures containing 0.6 μM calpain was initiated by the addition of $CaCl_2$ and terminated at various times, starting at about 1 s, by the addition of SDS sample buffer with 50 mM EGTA and boiling for 5 min. The samples were run on SDS/PAGE, gels were stained with Coomassie Brilliant Blue and band intensities were determined by densitometry. The degree of calpain autolysis was calculated by dividing the intensity of the 80 kDa form by the sum of intensities of the 76, 78 and 80 kDa bands. The apparent first-order rate constant of the autolysis was determined by fitting a linear function to the logarithm of these values plotted as a function of incubation time.

Other methods

SDS/PAGE was typically run on homogeneous gels in the buffer system described by Laemmli [25]. The intensities of the Coomassie-stained bands were determined by densitometry with a Bio-Rad GelDoc 1000 video-densitometer. Optical density readings were found to be proportional to protein quantity in the range examined, as checked by calibration curves. Protein determination was done as described in [26]. Data were evaluated by the MicroCal Origin technical graphics and data analysis software.

RESULTS

Autolysis of μ-calpain apparently precedes substrate hydrolysis in assays where casein degradation is compared with conversion of the large subunit [7,14]. In contrast, when we used MAP2, a far more sensitive calpain substrate than casein, its cleavage, as monitored by the disappearance of the 280 kDa band on SDS/PAGE (Figure 1), was much faster than μ-calpain autolysis: 50% conversion of MAP2 occurred at 12 s, whereas that of μ-calpain occurred only at about 9 min (Figure 2). It should be noted, however, that such comparisons of time courses of percentage conversion of an enzyme and its substrate may be fallacious: the relation certainly depends on the amount and quality of the substrate.

To assess whether MAP2 is broken down by autolysed or unautolysed μ-calpain, we measured the initial rate of MAP2 cleavage by μ-calpain autolysed to different extents, under conditions where the progress of autolysis is negligible during
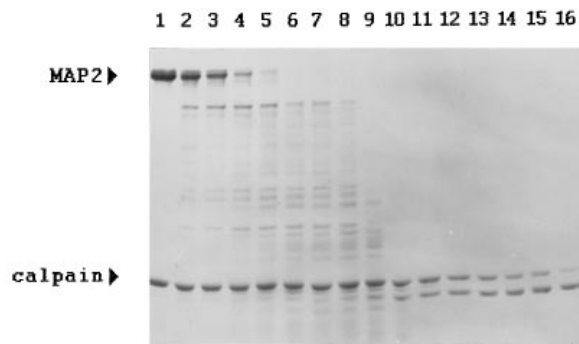
Figure 1   SDS/PAGE of MAP2 degradation and autolysis of μ-calpain at 10 μM Ca^{2+}

μ-Calpain (0.6 μM) and 0.5 μM MAP2 were mixed in calpain buffer with 1 mM CaCl$_2$ at 0 °C, aliquots were withdrawn at the times given below, treated with SDS sample buffer and run on a 6% (w/v) gel. The gel was stained with Coomassie Brilliant Blue. Determination of free Ca^{2+} concentration was as described in the Experimental section. Incubation times for lanes 1 through 16 were: 0 s (control, without added Ca^{2+}), 11 s, 17 s, 27 s, 37 s, 50 s, 54 s, 72 s, 2 min, 4 min, 8 min, 15 min, 25 min, 35 min, 50 min and 65 min respectively.
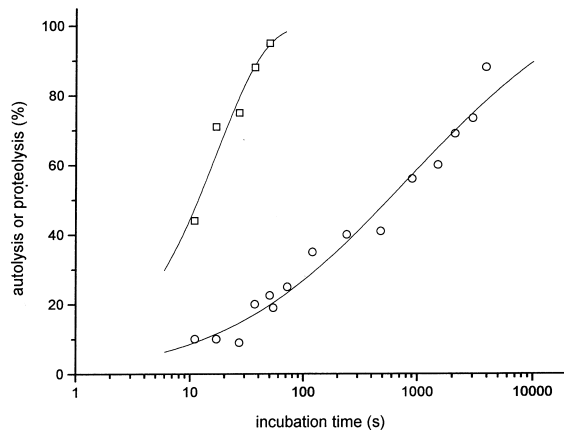

Figure 2   Time course of MAP2 disappearance and autolysis of μ-calpain large subunit

The intensities of the MAP2 and μ-calpain bands shown in Figure 1 were determined by densitometry. MAP2 proteolysis ($\square$) is given as the MAP2 converted as a percentage of the control value. The degree of μ-calpain autolysis ($\bigcirc$) is expressed as the intensity of the 76 kDa band as a percentage of the sum of intensities of the 76, 78 and 80 kDa bands.

Figure 3   Dependence of the initial rate of MAP2 hydrolysis on the degree of μ-calpain autolysis

MAP2 (0.8 μM) was added to 0.35 μM μ-calpain preincubated in calpain buffer with Ca^{2+} for various periods and the reaction was stopped by EGTA. The initial rate of MAP2 proteolysis, determined after SDS/PAGE as described in the Experimental section, was plotted as a percentage of the 76 kDa form at 10 μM ($\square$) and 300 μM ($\bigcirc$) free Ca^{2+} concentrations. The initial rate of MAP2 proteolyis extrapolated to unautolysed μ-calpain is zero within experimental error. Data represent means $\pm$ S.D. for three independent measurements.

(197 μM Ca^{2+}), and the maximum value was 1.52 s^{-1} at 30 °C (Figure 5). Secondly, we measured the Ca^{2+} dependence of μ-calpain activity in the conventional caseinolytic assay [23] with both native and pre-activated μ-calpain (Figure 5). It is conspicuous that the half-maximal activity with the pre-activated enzyme requires almost three orders of magnitude less Ca^{2+} (pCa^{2+} = 6.22) than autolysis. The Ca^{2+} requirement of native μ-calpain was somewhat higher (pCa^{2+} = 5.41), in accordance with the literature claiming that autolysis (activation) sensitizes the enzyme to Ca^{2+}. It should be pointed out, however, that the curve for native μ-calpain has no clear physical meaning, as it reflects the superimposition of Ca^{2+} sensitivities of autolysis/activation and casein hydrolysis.

Figure 4   Time course of μ-calpain autolysis

The disappearance of the 80 kDa form (as a percentage of the sum of the 76, 78 and 80 kDa forms) of 0.6 μM μ-calpain was determined as described in the Experimental section. The slopes of the straight lines fitted to the logarithm of these values at 10 ($\diamond$), 100 ($\triangle$), 250 ($\bigcirc$) and 1000 ($\square$) μM Ca^{2+} yield the apparent first-order rate constants of autolysis.

substrate hydrolysis. Such an experiment is feasible with MAP2, a highly sensitive substrate, but not with casein, a much poorer substrate. As seen in Figure 3, the rate of MAP2 proteolysis plotted against percentage conversion into the 76 kDa forms gives straight lines at both 10 and 300 μM Ca^{2+}, with different slopes but both intersecting the origin within the limits of error. This result shows that native, unautolysed μ-calpain (with intact 80 kDa large subunit), is a practically inactive proenzyme, and catalytic activity develops in strict proportion to the 76 kDa autolysed form. Thus it is difficult to interpret native μ-calpain activity unless we invoke the autolytic process.

To clarify the situation we first determined the Ca^{2+} dependence of μ-calpain autolysis. The disappearance of the 80 kDa form followed first-order kinetics at various Ca^{2+} concentrations over the major part of the reaction (Figure 4), suggesting that under these conditions autolysis is mainly intramolecular (compare [9]). The half-maximal rate constant was measured at pCa^{2+} = 3.7
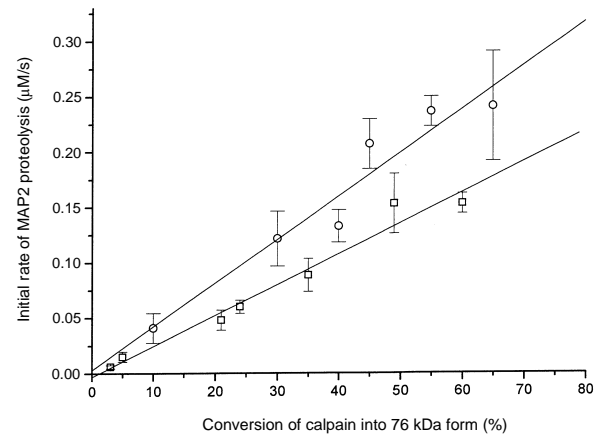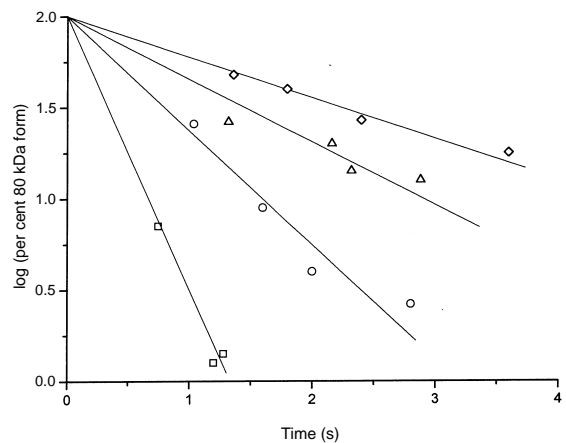
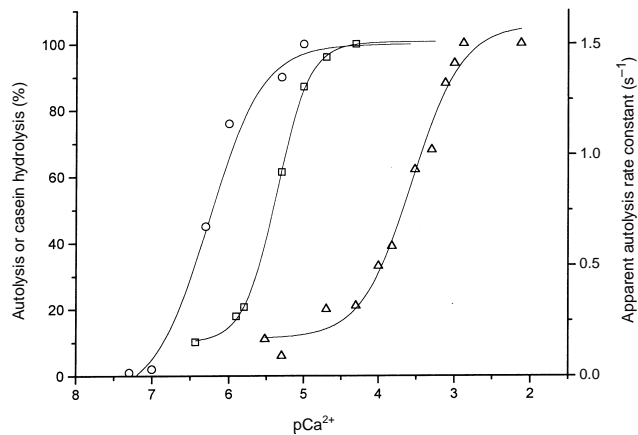**Figure 5   Effect of Ca²⁺ concentration on μ-calpain autolysis and activity**

The apparent first-order rate constant of μ-calpain autolysis (△, compare with Figure 4) and the initial rates of casein hydrolysis with 76 kDa μ-calpain (○) and native μ-calpain (□) were determined as described in the Experimental section. The half-maximal value of autolysis is at 197 μM Ca²⁺; half-maximal activity values with native and 76 kDa μ-calpain were at 3.9 and 0.6 μM Ca²⁺ respectively.
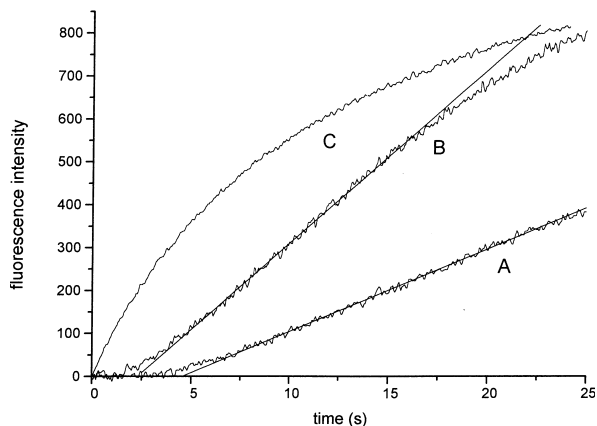

Figure 6   Progress curve of substrate degradation by μ-calpain measured with DTAF–MAP2

Degradation of 0.2 μM DTAF–MAP2 was initiated by the addition of 0.02 μM μ-calpain at 8 μM (**A**) and 28 μM (**B**) Ca²⁺ and by pre-autolysed μ-calpain at 28 μM Ca²⁺(**C**). Transit times of activation were determined by fitting a straight line to the linear phase of progress curves and extrapolating them to zero substrate conversion.

In a third set of experiments we attempted to determine the Ca²⁺ dependence and maximum rate of activation of μ-calpain. This was rendered possible by using our novel, continuous calpain assay with fluorescently labelled MAP2, which enabled us to monitor even the first seconds of the reaction. As seen in Figure 6, there is a lag phase ('transit time'), decreasing with increasing Ca²⁺ concentration, in the time course that corresponds to activation. If we assume that activation can be described by a first-order reaction, the reciprocal of transit time gives the first-order rate constant of activation [24]. Its Ca²⁺ dependence (Figure 7) reveals half-maximal activation at pCa²⁺ = 3.8 (158 μM Ca²⁺) and the maximum is attained at 2.15 s⁻¹. These values agree remarkably well with the corresponding parameters of autolysis described above (pCa²⁺ = 3.7, maximum
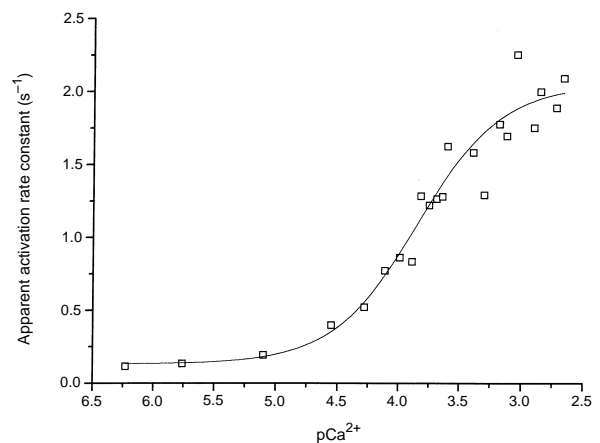

Figure 7   Ca²⁺ dependence of the μ-calpain activation rate constant

The apparent first-order rate constant of μ-calpain activation (reciprocal of transit times determined as in Figure 6) is plotted as a function of free Ca²⁺ concentration. The half-maximal rate of activation is at 158 μM Ca²⁺. Data points represent averages of duplicate experiments.

$k_{\mathrm{I}} = 1.52$ s⁻¹), in view of the entirely different techniques that were used to derive them. We can conclude that in our conditions autolysis and activation run in close parallel.

DISCUSSION

The mode of μ-calpain activation is rather controversial in the literature. Until recently, two opposing views existed: one held that native μ-calpain is an inactive proenzyme that becomes activated by autolysis [3,4], whereas according to the other, native μ-calpain is fully active and only its Ca²⁺ sensitivity is increased upon autolysis [15,16]. Recently, however, it has been proposed that activation is effected by subunit dissociation [5,6], autolysis being unnecessary for, or at best a preliminary to, the critical dissociation step. This dissociation model, although introducing a new mechanistic step, does not resolve the earlier controversy. The major reason for the ambiguity is that most previous arguments can be challenged on a strictly kinetic basis.

We illustrated this point by scrutinizing the canonical argument in favour of the inactive proenzyme nature of native μ-calpain, i.e. the observation that substrate (casein) hydrolysis lags behind autolysis [7,14]. We could show that at low concentrations of a much more sensitive substrate (MAP2) the picture is reversed: autolysis lags behind substrate hydrolysis. These apparently opposite behaviours of casein and MAP2 might be interpreted in terms of a true difference between their degradation mechanisms, i.e. that they are acted on by differently autolysed states of the enzyme. However, it can be shown that this is a kinetic, rather than a mechanistic, phenomenon that stems from the difference in sensitivity and concentration of the substrates. We demonstrated this by measuring the initial rate of enzyme activity and correlating it with one of the enzyme forms. We found that the rate of MAP2 hydrolysis was a linear function of the amount of the 76 kDa form and at zero autolysis activity was also practically nil even at saturating Ca²⁺ concentrations. This finding strongly suggests that native μ-calpain is inactive, at least in a sense that it does not hydrolyse an exogenous substrate molecule before its intramolecular autolysis. In strictly molecular terms, of course, calpain must already be active before the autolytic reaction, otherwise this reaction would not take place either and

native calpain could not be inhibited by activity-dependent inhibitors such as E-64 [13] or leupeptin [27] in the presence of Ca^{2+}. Two alternative interpretations of this phenomenon are that the active site of μ-calpain is blocked by its autoinhibitory N-terminal segment and so autolysis is truly a prerequisite for hydrolysis of a substrate molecule, or that the scissile bond within the N-terminal segment is so ideally positioned for cleavage that no exogenous substrate can compete with it although its removal is not really needed for activation. To distinguish between these alternatives novel approaches are required.

The inactive proenzyme nature of μ-calpain is further supported by the appearance of a lag phase in the progress curves of MAP2 hydrolysis that only disappears if calpain is pre-autolysed, and also by the observation that autolysis and activation rate constants are practically equal and display identical Ca^{2+} dependences. This good match argues strongly that autolysis is inevitably accompanied by activation and that substrate hydrolysis, at least on a macroscopic scale, cannot precede autolysis. At variance with this finding, the dissociation model [5] states that at Ca^{2+} concentrations comparable with that required for autolysis calpain dissociates into subunits and becomes active without further autolysis. This model originates from observing dissociation in the presence of inhibitors, which can hardly be an obligatory step towards the activation of the enzyme as it would preclude autolysis, which does occur both *in vitro* [3,9,12,14,16] and *in vivo* [28,29]. It is more likely that dissociation is a consequence of autolysis (compare [6]) but it will take further studies to elucidate whether it is a prerequisite of activation. For lack of contrary evidence it seems conceivable that the 76/30 kDa or 76/18 kDa dimeric form of μ-calpain is already active before its dissociation, which might thus be a consequence and not a cause of activation. The result that activation is kinetically inseparable from autolysis under our conditions is compatible with the latter assumption; it implies that dissociation is either a fast (not rate-limiting) process or unnecessary for activation. Of course, it cannot be excluded that under different conditions dissociation is indeed an important mechanistic step in activation.

A further intriguing issue is the high Ca^{2+} requirement for μ-calpain activation (158-197 μM), observed independently in the autolysis and activation studies. Such a high value has been already hinted at in the literature [8,16], but this is the first time it has been precisely measured in kinetic experiments. Autolysed calpain, in agreement with the literature, is more sensitive to Ca^{2+} (0.6 μM), whereas the Ca^{2+} sensitivity of 'native μ-calpain' activity exhibits an intermediate value (3.9 μM). However, the latter value represents an ill-defined temporal average of the Ca^{2+} sensitivities of autolysis of the native enzyme and activity of the autolysed enzyme. Therefore the true Ca^{2+} requirement of autolysis, the initial step in calpain activation, is indeed very high. We should bear in mind, however, that several factors have been identified that potentiate autolysis by sensitizing calpain to Ca^{2+}. These include phospholipids [16,30], the most effective of which is phosphatidylinositol, which increases Ca^{2+} sensitivity 10-fold, activator proteins [31,32], with as large an effect as 100-fold, and DNA [33], which has been shown to sensitize m-calpain to Ca^{2+} about 100-fold. In accordance with these observations, autolysis does occur under physiological conditions [28,29]. The high Ca^{2+} requirement of the isolated enzyme might have evolved to ensure that in the absence of these activators physiological Ca^{2+} signals do not activate μ-calpain, which would be deleterious

for the cell. The importance of low Ca^{2+} sensitivity is brought out in comparison with the significantly higher Ca^{2+} affinity of calmodulin (K_d 0.1–1 μM [34]), which gave rise to the ancestral calpain molecule by fusing with a protease domain [35]. Ca^{2+} binding of calpain must have evolved to temper the Ca^{2+} affinity of the calmodulin domain, probably to ensure tight control of activation owing to the requirement of interaction with both activators and Ca^{2+}.

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