The roles of N-terminal autolysis of the large (80 kDa) and small (28 kDa) subunits in activation of rat m-calpain, in lowering its Ca\textsuperscript{2+} requirement, and in reducing its stability have been investigated with heterodimeric recombinant calpains containing modified subunits. Both autolysis and [Ca\textsuperscript{2+}]\textsubscript{0.5} were influenced by the ionic strength of the buffers, which accounts for the wide variations in previous reports. Autolysis of the small subunit (from 28 to 20 kDa) was complete within 1 min but did not alter either the Ca\textsuperscript{2+} requirement ([Ca\textsuperscript{2+}]\textsubscript{0.5}) or the stability of the enzyme. Autolysis of the NH\textsubscript{10}– 80 kDa large subunit at Ala\textsuperscript{9}–Lys\textsuperscript{10} is visible on gels, was complete within 1 min, and caused a drop in [Ca\textsuperscript{2+}]\textsubscript{0.5} from 364 to 187 \mu M. The lower value of [Ca\textsuperscript{2+}]\textsubscript{0.5} is therefore a property of the \textless 9–80 kDa large subunit. Autolysis at Ala\textsuperscript{9}–Lys\textsuperscript{10} of the unmodified 80-kDa large subunit is not detectable on gels but was assayed by means of the fall in [Ca\textsuperscript{2+}]\textsubscript{0.5}. This autolysis was complete in 3.5 min and was inhibited by high [NaCl]. The autolysis product of these calpains, which is essentially identical to that of natural m-calpain, was unstable in buffers of high ionic strength. Calpain in which the large subunit autolysis site had been mutated was fully active but did not undergo a drop in [Ca\textsuperscript{2+}]\textsubscript{0.5}, showing that m-calpain is active prior to autolysis. The main physiological importance of autolysis of calpain is probably to generate an active but unstable enzyme, thus limiting the in vivo duration of calpain activity.

The calpains (EC 3.4.22.17) are cytoplasmic cysteine proteinases, which are thought to be regulated by means of their Ca\textsuperscript{2+} dependence. While much work has been done on their biochemical properties, many aspects of autolysis, activation, and Ca\textsuperscript{2+} requirement remained unresolved (1–3). Two mammalian forms, \mu- and m-calpain (calpain I and II), have been most studied, since they can be isolated from animal tissues. Some other calpain forms are known so far only from their mRNA (3, 4), and the chicken calpains are not further considered here since they appear to be slightly different (5, 6). Complete purification of calpain in adequate amounts from tissue extracts is difficult, and a bacterial expression system has been described, which with the aid of a His-tag provides larger amounts of pure enzyme in about 3 days and provides a means for mutational and structural work (7). A baculovirus-based expression system has also been reported (8).

The calpains consist of an 80-kDa catalytic subunit (the large subunit), containing four domains, I–IV, and a 28-kDa regulatory subunit (the small subunit), containing two domains, V and VI (9, 10). Domains I and V are involved in autolysis; domain II contains the most obvious active site residues (11); the function of domain III is not yet known, although it must also take part in the conformational changes induced by Ca\textsuperscript{2+} and the C-terminal domains of both subunits, IV and VI, contain putative E-F hand motifs, some of which bind Ca\textsuperscript{2+} (12–15). No structural information is available for the complete calpain heterodimer, but the crystal structures of domain VI, with and without bound Ca\textsuperscript{2+}, have been solved recently.\footnote{1} On exposure to sufficient Ca\textsuperscript{2+}, the calpains are assumed to undergo a conformational change which permits the following four events: limited autolysis of the small subunit from 28 to 20 kDa; limited N-terminal autolysis of the large subunit; proteolysis of a substrate such as casein if it is present; and further inactivating proteolysis of the large subunit. However, the order of these events and the Ca\textsuperscript{2+} concentrations required have been difficult to establish. The Ca\textsuperscript{2+} requirements ([Ca\textsuperscript{2+}]\textsubscript{0.5}) for casein hydrolysis by (initially) non-autolyzed calpains are usually reported to lie in the ranges of 5–50 \mu M Ca\textsuperscript{2+} for \mu-calpain and 250–1000 \mu M Ca\textsuperscript{2+} for m-calpain. These ranges are lowered by prior autolysis to 1–5 \mu M Ca\textsuperscript{2+} for \mu-calpain and 100–200 \mu M Ca\textsuperscript{2+} for m-calpain (16–25). The reported values vary widely for at least two reasons, first because the casein assay reflects the net effects of activation, autolysis, and inactivation, and is not well suited for kinetic studies (26); and second because, as shown here, the observed values are highly dependent on experimental conditions.

While it is fairly clear that autolysis lowers [Ca\textsuperscript{2+}]\textsubscript{0.5} of both \mu- and m-calpain, it has not been clear whether large and/or small subunit cleavage is required for, or responsible for, the fall in [Ca\textsuperscript{2+}]\textsubscript{0.5} and whether the non-autolyzed calpains are intrinsically active against substrates such as casein. In this work, we have answered some of these questions about autolysis and Ca\textsuperscript{2+} requirement in m-calpain, using a variety of recombinant large and small subunits with N- and C-terminal modifications.

**EXPERIMENTAL PROCEDURES**

*Escherichia coli* strain BL21(DE3) and the plasmids pET-16b(+) , pET-20b(+) , and pET-24d(+) were obtained from Novagen Inc., Madison, WI. pTRXFUS was a generous gift of Dr. LaVallie (see LaVallie et al. (27)). The plasmid pCApET-24, used for expression of small subunits, has been described previously (7).

**N-terminal Amino Acid Sequencing**—All of the subunit modifications described were checked by DNA sequencing, and in some cases at the amino acid level. Amino acid sequences were obtained by automated Edman degradation, either from individual subunits following gel electrophoresis and blotting onto polyvinylidene difluoride membrane or from calpain solutions containing both subunits following concentration on a ProSpin cartridge (Applied Biosystems).

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\footnote{1} Dr. M. Cygler, personal communication.
Autolysis, Ca2+ Requirement, and Stability in m-Calpain

Calpain Large Subunit—cDNA for the rat m-calpain 80-kDa subunit and for this subunit with a C-terminal His-tag (80k-CHis6), in the plasmid pET-24d (+), has been described previously (7). Natural calpain large subunits are N-terminally blocked, presumably with an acetyl group, but in E. coli the subunits are expressed unblocked, with or without a C-terminal His-tag.

The 360-bp fragment coding as the 21-kDa subunit, is now referred to as the first autolysis product of m-calpain, shown by amino acid sequencing. This truncation was designed to approximate the first autolysis product of m-calpain, shown by amino acid sequencing. The results of a single experiment are shown, but most expressions were repeated with consistent results. The specific activity is given for the enzyme after three purification steps. Construction of the various large subunits is described under “Experimental Procedures,” and the small subunit Δ86 and NHis10-28k have been described previously. For comparison, an initial yield of 1,350 units of natural rat m-calpain was obtained from 400 g of rat carcass.

| Large subunit | Small subunit | Initial yield | Specific activity |
|---------------|---------------|---------------|------------------|
| 80k           |               | Δ86           | 18,000           | 1,880            |
| 80k-CHis6     |               | Δ86           | 36,000           | 1,860            |
| NHis10-80k    |               | Δ86           | 7,000            | 1,780            |
| NHis10-80k-CHis6 |         | Δ86           | 36,000           | 1,800            |
| LSF-Δ8,9F-80k-CHis6 |   | Δ86           | 7,500            | 200              |
| LSS,Δ8,9F-80k-CHis6 |   | Δ86           | 25,000           | 1,800            |
| NHis10-28k    |               | 18,900        | 1,400*           |
| NHis10-28k    |               | 23,600        | 1,720            |
| TRX-Δ80k-CHis6 |               | 200           | 0                |
| TRX-Δ80k-CHis6 |               | 690           | 0                |

* Not fully purified.

# Not fully purified.

Tris-HCl, pH 7.6, net final CaCl2 concentrations from zero μM to 3.8 mM, and 20 μl of enzyme sample (containing in most cases 5–7 units of activity). The mixtures were incubated at 25 °C for 30 min; reaction was terminated by addition of 0.35 ml of ice-cold 10% trichloroacetic acid, and the A280 nm values of the supernatants were recorded. For Ca2+ titration, the enzymes were always re-purified by chromatography on MonoQ in buffers containing 50 mM Tris-HCl and 2 mM EDTA. Precise comparisons were valid only between enzymes purified on the same day and titrated in the same buffers. The dissociation constant for Ca2+-EDTA at pH 7.6 was taken to be 1.22 × 10−6 M. The Ca2+ concentration required for half-maximal activity with casein as substrate is given as [Ca2+]H50. This value was calculated by fitting the normalized activity data to the equation y = x/[Ca2+]H50 + x, where y is the fraction of maximum activity, k = [Ca2+]H50 n is the Hill constant, and x is [Ca2+].

Conditions of Autolysis—Calpain samples (0.5–2 μM, 50–200 μg/ml) in 2 mM EDTA, 10 mM β-mercaptoethanol, 0.05–0.5 mM NaCl, 50 mM Tris-HCl, pH 7.6, at 20 °C were adjusted with CaCl2 to a net final concentration of 2.5 mM Ca2+, and shaken, normally for 30–120 s, followed by addition of EDTA to a net final concentration of 2 mM free EDTA. In some cases, casein (5 mg/ml) was also present. Control samples received the same volumes of EDTA first and CaCl2 second. Samples were taken for Ca2+ titration, gel electrophoresis, and blotting, or re-chromatography on a MonoQ column. Autolysis conditions were chosen so that not more than 25% of the original activity was lost.

Stability of Autolysis Products—Stability of calpain is defined here in a special sense, in terms of recovery of activity from the MonoQ column. Control and autolysed calpain samples were assayed to determine the loss of total activity due to autolysis and then chromatographed on the MonoQ column in the presence of 2 mM EDTA. Non-autolysed m-calpains were eluted in high yield from this column at approximately 0.4 mM NaCl, but for some constructs the activity remaining after brief autolysis was recovered only in poor yield from the column.

**RESULTS**

Effects of Subunit Modifications on Formation of Active Heterodimeric Calpains

Many combinations of modified m-calpain large and small subunits have been co-expressed. The yields of activity derived from 4 liters of E. coli, as observed after the first purification step, are given in Table I. The table also shows the specific activities of the purified products, which with one or two exceptions were all close to 1800 units/mg protein. The subunit compositions of the purified calpains were checked by immunoblotting and by Coomassie staining of SDS/Tris-Tricine gels.

2 The abbreviations used are: bp, base pair(s); TRX, thioredoxin; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

3 Elce, J. S., Davies, P. L., Hegadorn, C., Maurice, D. H., and Arthur, J. S. C. (1997) Biochem. J., in press.
to establish their heterodimeric character. The differing yields of activity in each co-expression experiment appear to reflect primarily the relative levels of expression of the two subunits, but the fairly constant specific activities of the purified products show that the subunit modifications in most cases did not affect heterodimer assembly or catalytic function. However, some of the active heterodimers had properties that were useful in the study of autolysis and [Ca$^{2+}$]$_{0.5}$. Much of the work has been done with calpains having a C-terminal His-tag, since they were more highly expressed, but the results have been duplicated in several cases with calpains lacking the C-terminal His-tag. Table I also includes the fully active construct LH8, A9F-80k-CHis$_6$/Δ86, in which the autolysis site at Ala$^9$Lys$_{10}$ has been abolished, for comparison with LH8, A9F-80k-CHis$_6$/Δ86, which was surprisingly inactive.

Steric Factors in Co-expression

As described elsewhere, both the unmodified 28-kDa or NHis$_{10}$-28k were less well expressed than Δ86 but were able to form active enzymes with 80 kDa. Conversely, NHis$_{10}$-80k formed active enzyme with Δ86. However when both large and full-length small subunits contained N-terminal extensions (NHis$_{10}$-80k/NHis$_{10}$-28k or TRX-80k-CHis$_6$/NHis$_{10}$-28k), very little activity was formed and was not stable to further purification (Table I). Immunoblots of the crude extracts showed that the subunits were sufficiently expressed, and particularly the TRX-construct was highly expressed as predicted (not shown) (27). These results indicate that the two N-terminal extensions caused a steric interference to heterodimer formation and suggest therefore that the N termini of both subunits must be close to each other in normal m-calpain. It may be noted here also that the structure of domain VI strongly suggests that the C termini of the large and small subunits are also close together.

Measurement of Ca$^{2+}$ Requirement for Casein Hydrolysis

Fig. 1 shows measurements of [Ca$^{2+}$]$_{0.5}$ of 80k-CHis$_6$/Δ86 for casein hydrolysis, as a function of the concentration of NaCl and of Tris buffer at pH 7.6. The effects of KCl were identical to those of NaCl. The increases in buffer and salt concentrations raised the value of [Ca$^{2+}$]$_{0.5}$ progressively from 175 to 436 μM. A very similar dependence of [Ca$^{2+}$]$_{0.5}$ on ionic strength was observed with several other recombinant calpains that were tested and also with partially purified natural rat m-calpain. Variation of pH between 7.2 and 7.8 (around the pH optimum for casein hydrolysis (30)) had little effect, but the value of [Ca$^{2+}$]$_{0.5}$ was greatly increased at pH 8.5 (not shown). The Ca$^{2+}$ concentrations of the solutions were checked by flame photometry, and we confirmed the observation (19) that dialysis of the casein substrate against EDTA had no effect on [Ca$^{2+}$]$_{0.5}$ values. All subsequent Ca$^{2+}$ titrations were therefore performed in 50 mM Tris-HCl, 0.2 mM NaCl, pH 7.6.

Small Subunit Autolysis, [Ca$^{2+}$]$_{0.5}$, and Heterodimer Stability

Expression of m-calpain with an NHis$_{10}$-28k small subunit (in fact a mixture of partially degraded small subunits) and autolysis of this heterogeneous small subunit to a homogeneous 20-kDa small subunit have been described previously. In 80k-CHis$_6$/Δ86, small subunit autolysis was complete within 1 min (as shown later, Fig. 4) and did not cause a fall in [Ca$^{2+}$]$_{1.0}$ (Fig. 2a). Since this autolysis was done in 0.4 M NaCl, as discussed later, large subunit autolysis can be neglected in this experiment; the result therefore demonstrates that small subunit autolysis alone does not contribute to the fall in [Ca$^{2+}$]$_{1.0}$ in this m-calpain.

The same conclusion can be drawn from a separate experiment. The recombinant Δ86 small subunit and the natural 20-kDa autolysis product of the small subunit are essentially identical (31). 80k-CHis$_6$/Δ86 therefore closely resembles the product expected if only the small subunit were autolyzed on exposure of 80k-CHis$_6$/Δ86 to Ca$^{2+}$, and there was no significant difference in [Ca$^{2+}$]$_{0.5}$ between 80k-CHis$_6$/Δ86 and non-autolyzed 80k-CHis$_6$/NHis$_{10}$-28k (Fig. 2b).

Intermolecular Autolysis of the Small Subunit

To investigate the relative contributions of inter- and intramolecular reaction to autolysis of the small subunit, autolysis experiments were performed with mixtures of active and inactive calpains. In this experiment only, the full-length small subunits were from rabbit, and it should be noted that the monoclonal antibody (kindly provided by Dr. R. Mellgren, University of Toledo) used to observe the small subunit does not react with the rat small subunit. No other difference has been detected between calpains composed of the rat m-calpain large subunit and the natural, rabbit or rat calpain small subunits. As shown in Fig. 3, in a mixture of equal molar amounts of the inactive mutant C105S-80k-CHis$_6$/rabbit-NHis$_{10}$-28k with active 80k-CHis$_6$/Δ86, the heterogeneous small subunits of the inactive calpain were converted to a homogeneous 20-kDa form within 1 min, and this reaction could only be intermolecular (tracks 1–3). Autolysis of the small subunit in active 80k-CHis$_6$/rabbit-NHis$_{10}$-28k was also complete within 1 min (tracks 4–6), but in this case it is not possible to distinguish between inter- and intramolecular reaction. In the control experiment, C105S-80k-CHis$_6$/rabbit-NHis$_{10}$-28k alone was not autolyzed (tracks 7–9).

Effect of Casein on Subunit Autolysis

Fig. 4 shows the autolysis for 0–8 min in 0.25 M NaCl with and without casein of (a) the large subunit in NHis$_{10}$-80k/Δ86

\[ y = \frac{kn}{x^n} \]

5 J. S. Elce, unpublished work.
and (b) the heterogeneous small subunit in 80k-CHis6/NHis10-28k. In each case it is evident that autolysis was slightly retarded by the presence of 5 mg/ml casein (but was still essentially complete after 1 min), which is consistent with some contribution by intermolecular reaction to autolysis of both subunits. This effect of casein on autolysis of the large subunit was evident also in the Ca^{2+} titrations described below.

Large Subunit Autolysis and [Ca^{2+}]_{0.5}

In the study of large subunit autolysis, conditions were chosen so that the calpains did not lose more than 25% of their starting activity, since it was felt that normalization of Ca^{2+} titration data obtained after very extensive autolysis might be misleading. It was found that the effects of autolysis of the various calpains on their [Ca^{2+}]_{0.5} for casein hydrolysis depended both on the experimental conditions during autolysis and also on the differences in the recombinant large subunits.

Effect of High [NaCl]—Autolysis of 80k/Δ86 in 0.4 M NaCl (as eluted from the Q-Sepharose column, the final step in purification) caused loss of activity, showing that the calpain was active, but did not change [Ca^{2+}]_{0.5}. The titration curves are not shown since they were identical to those in Fig. 2. This result was unexpected but was highly reproducible. The activity remaining after autolysis of 80k-CHis6/Δ86 in 0.4 M NaCl was recovered in high yield from re-chromatography on MonoQ, and this material had clear N-terminal sequence showing that both subunits were intact. This result shows that m-calpain is active without large subunit autolysis, a point that was established even more rigorously with the L8F, A9F mutant (see below). Partially purified natural rat m-calpain also showed no change in [Ca^{2+}]_{0.5} on autolysis in 0.4 M NaCl (data not shown).

When 80k/Δ86 was autolysed in 0.18 M NaCl, the value of [Ca^{2+}]_{0.5} showed the expected fall; in the presence of 5 mg/ml casein, about 270 s of autolysis were required to reach the lowest value of [Ca^{2+}]_{0.5}, falling from 324 to 186 μM (Fig. 5a). A comparison of autolysis of 80k/Δ86 for 120 s in 0.18 M NaCl with and without casein showed that casein slightly retarded the conversion to a low-Ca^{2+} requiring form, from 338 μM at time 0 to 292 μM in the presence of casein and to 246 μM in the absence of casein (Fig. 5b). N-terminal sequencing of 80k-
Autolysis, Ca$^{2+}$ Requirement, and Stability in m-Calpain

CHis$_{10}$/Δ86 after incubation with Ca$^{2+}$ for 10 min gave a mixture of three sequences, representing approximately 33% intact large subunit (AGIAMKL), 66% autolyzed large subunit (KDREAA), and 100% intact small subunit. This cleavage at Ala$_9$-Lys$_{10}$ is in agreement with previous reports (23).

Effect of the N-terminal His-tag—In contrast to the behavior of 80k/Δ86, the fall in [Ca$^{2+}$]$_{0.5}$ on autolysis of the N-terminally extended NHis$_{10}$-80k/Δ86 at Ala$_9$-Lys$_{10}$ was equally rapid in high or low NaCl, or with or without casein (Fig. 6), and the small drop in molecular mass of the large subunit could be readily detected on gels (see above, Fig. 4). The large subunit of autolyzed NHis$_{10}$-80k/Δ86 was N-terminally sequenced after gel electrophoresis and blotting, giving the sequence KDREAA, which results from cleavage at Alas$_9$-Lys$_{10}$ (strictly Alas$_{30}$-Lys$_{31}$).

Over longer periods of autolysis, the remaining activity correlated with densitometry of the large subunit (Fig. 7), and since the large subunit after 30–60 s is 100% cleaved at Alas$_9$-Lys$_{10}$, the activity after this time was therefore clearly a property of the autolyzed calpain. These results show that the lower [Ca$^{2+}$]$_{0.5}$ in m-calpain is a property of the Δ9–80k large subunit.

Effect of Abolishing the Autolysis Site—Calpain does not possess a strong consensus cleavage sequence to direct our choice of mutation, but there are very few examples of cleavage with a phenylalanine residue in the P1 position (1). In accordance with this, the autolysis site mutant L8F, A9F-80k/Δ86 showed no significant change in [Ca$^{2+}$]$_{0.5}$ under any autolysis conditions (Fig. 8). This result demonstrates clearly, as also suggested earlier, that calpain with an intact large subunit is active in casin hydrolysis without autolysis.

Large Subunit Autolysis and Calpain Stability

After quenching the autolysis reaction with excess EDTA, the remaining activity of the various calpains was stable for some hours at 0 °C. The results in Table III suggest, however, that the autolysis product Δ9–80k/Δ86 is relatively unstable during purification on MonoQ, which includes elution at approximately 0.4 M NaCl. Incubation of 80k-CHis$_{10}$/Δ86 with 2.5 mM Ca$^{2+}$ in 0.4 M NaCl causes loss of activity with time but does not involve cleavage at Alas$_9$-Lys$_{10}$, and the subsequent MonoQ recovery was high (84%); autolysis of several constructs in conditions where a relatively slow fall in [Ca$^{2+}$]$_{0.5}$ occurs and where the conversion to Δ9–80k/Δ86 was therefore incomplete was followed by intermediate recoveries from MonoQ (40–60%); and autolysis of NHis$_{10}$-80k-CHis$_{10}$/Δ86, which was complete, gave much lower recoveries (20%). In the latter case, it was shown that the active material recovered in low yield from MonoQ was Δ9–80k-CHis$_{10}$/Δ86 (Fig. 9).

N-Terminal Truncation of the Large Subunit in m-Calpain

The treatment of the initiating methionine residue in E. coli does not permit expression of the previously suggested large subunit autolysis products, Δ9–80k and Δ19–80k, with the...
FIG. 7. Correlation of NHis10–80k/Δ86 activity with remaining large subunit protein. NHis10–80k/Δ86 was incubated with 2 mM Ca\(^{2+}\) and samples were taken and quenched with EDTA at times up to 8 min. These were assayed for activity with casein (○), and the amount of large subunit protein remaining was estimated by densitometry of a Coomassie-stained gel (●). It is known (see Fig. 4) that after about 45 s the large subunit has been completely cleaved at Ala9–Lys10.

FIG. 8. Absence of large subunit autolysis in L8F, A9F–80k-CHis6/Δ86. Autolysis was performed for 120 s in several different conditions, followed by measurement of N-terminal truncations of the large subunit. The N-terminal amino acid sequences of the natural rat m-calpain and calpains constructs are no longer active (32, 33). The relative rates of limited N-terminal autolysis and of inactivating autolysis are also dependent on the construct and on the ionic strength.

FIG. 9. Autolysis of NHis10–80k/CHis6/Δ86 and recovery of autolysis products. The figure shows the 80-kDa region of a Coomassie-stained Tris glycine gel. The tracks contained 1 and 5, NHis10–80k-CHis6/Δ86 prior to autolysis; track 2, NHis10–80k-CHis6/Δ86 following autolysis in 0.18 mM NaCl for 120 s; track 3, non-autolyzed NHis10–80k-CHis6/Δ86 recovered from the MonoQ column; track 4, autolyzed NHis10–80k-CHis6/Δ86, i.e. Δ9–80k-CHis6/Δ86, recovered from the MonoQ column.

TABLE II

| N-terminal sequence | Abbreviation | Yield of activity |
|---------------------|--------------|------------------|
| AGIAMKLAKDREAAEGLGIAMKLAKDREA | 80k-CHis6 | 36,000 |
| GIANKLAKDREAAEGLGIANKLAKDREA | Δ8–80k-CHis6 | 16,000 |
| MKCLAKDREAAEGLGAMKLAKDREA | Δ4–80k-CHis6 | 0 |
| MOEREAMK89–80k-CHis6 | 0 |
| AKOREAMΔ8–80k-CHis6 | 0 |
| AHERAIKAΔ20–80k-CHis6 | 0 |

*These constructs were prepared with or without a C-terminal His-tag, which affected the yield of expressed protein but did not affect whether activity was observed.

**DISCUSSION**

At the beginning of this work we found it difficult to reconcile the extensive but sometimes inconsistent literature on [Ca\(^{2+}\)]\(_{0.5}\) and autolysis in m-calpain. It has now become clear that the observed values of [Ca\(^{2+}\)]\(_{0.5}\) are highly dependent on experimental conditions. It should be noted also in the following discussion that autolysis of calpain involves not only the generation of the Δ93-small subunit and the Δ9-large subunit, in which the enzyme is active and has a lower [Ca\(^{2+}\)]\(_{0.5}\), but also involves cleavage of the large subunit to smaller fragments that are no longer active (32, 33). The relative rates of limited N-terminal autolysis and of inactivating autolysis are also dependent on the construct and on the ionic strength. Together these observations appear to explain much of the wide variation in [Ca\(^{2+}\)]\(_{0.5}\) reported over the last 15 years from different laboratories.

**Conditions for Measurement of [Ca\(^{2+}\)]\(_{0.5}\)—With 80k-CHis\(_{d}\)△86, in the absence of any prior autolysis, it was found that [Ca\(^{2+}\)]\(_{0.5}\) was highly dependent on the concentration of Tris buffer and of NaCl (Fig. 1). This was true also for several different calpain constructs and for partially purified natural rat m-calpain. Within the range of pH 7.2–7.8, the effect of pH was not significant, but at higher pH values [Ca\(^{2+}\)]\(_{0.5}\) increased significantly. These effects of ionic strength and pH on [Ca\(^{2+}\)]\(_{0.5}\) for m-calpain have not, to our knowledge, been reported previously.

**Small Subunit Autolysis**—To consider first small subunit autolysis, we have shown that introduction of 80k-CHis\(_{d}\)/NHis\(_{10}\)-28k with Ca\(^{2+}\) caused rapid autolysis of the small subunit. This was easily seen on blots (Fig. 4) but did not alter [Ca\(^{2+}\)]\(_{0.5}\) (Fig. 2a). In this experiment, since autolysis was performed in 0.4 mM NaCl, the large subunit was not cleaved. The result was confirmed by the finding that 80k-CHis\(_{d}\)/NHis\(_{10}\)-28k and its hypothetical small subunit-only autolysis product, 80k-CHis\(_{d}\)/Δ86,
had identical \([\text{Ca}^{2+}]_{0.5}\) (Fig. 2b). These results demonstrate unequivocally that autolytic removal of domain V does not affect \([\text{Ca}^{2+}]_{1.05}\) of m-calpain. Domain V of the small subunit is clearly not required for calpain activity or for formation of active calpain as a foreign protein in *E. coli*, although it presumably has some role in calpain regulation in eukaryotic cells. It may be noted also that removal of domain V does not by itself cause instability in m-calpain, since 80k(\(±\text{CHis}_8\))/\(Δ86\) is stable through several steps of purification.

We have not studied the question of whether small subunit autolysis is strictly required, as seems likely, before large subunit autolysis or before casein hydrolysis. It would be interesting to attempt to generate mutant full-length small subunits that are resistant to autolysis to prove that removal of domain V is the essential first step. This may be difficult in practice, since domain V appears to be prone to hydrolysis at several sites.3

Small subunit autolysis has been described both as intramolecular, on the basis of kinetic observations (19, 34), and also as intermolecular (22, 35). The experiments reported here with inactive calpain showed that intermolecular autolysis of the small subunit can occur at least as rapidly as any other autolysis step in this work (Fig. 3). In active m-calpain, small subunit autolysis was slightly retarded in the presence of casein, which also suggests some contribution by an intermolecular mechanism (Fig. 4). While the intermolecular reaction is clearly feasible in *vivo*, intramolecular reaction may be more likely in *vivo*, where the calpains are “relatively dilute and surrounded by potential substrates” (35).

**Large Subunit Autolysis in m-Calpain**—It has frequently been reported that incubation of natural m-calpain with \(\text{Ca}^{2+}\) causes a fall in \(\text{Ca}^{2+}\)-requirement for hydrolysis of casein or other substrate, without any apparent change in the large subunit that can be detected on gels (2, 23, 24). As a result of this difficulty, the fall in \([\text{Ca}^{2+}]_{1.05}\) was at first attributed to small subunit autolysis alone (34) and later to cleavage of the large subunit either at Ala19-Ser20 (16) or at Ala9-Lys10 (23).

The construct NHHis10-80k/\(Δ86\) has the advantage that its autolysis can be easily seen on gels by Coomassie staining or by immunoblotting (Figs. 4, 9), in a manner highly reminiscent of autolysis in \(μ\)-calpain (22, 26, 36). It should be noted that the pre- and post-autolysis values of \([\text{Ca}^{2+}]_{1.05}\) are the same for NHHis10-80k/\(Δ86\) as for 80k/\(Δ86\) and that the autolysis product \(Δ9–80k/\(Δ86\) is identical to the autolysis product of natural m-calpain, except for 7 N-terminal amino acids in the small subunit that do not affect its properties.3 These results demonstrate that the lower \([\text{Ca}^{2+}]_{1.05}\) in m-calpain is a property of the \(Δ9–80k\) large subunit, so that the value of \([\text{Ca}^{2+}]_{1.05}\) can be used as a reliable index of large subunit autolysis at Ala9-Lys10.

Autolysis at Ala8-Lys14 in NHHis10-80k/\(Δ86\) is essentially complete in about 50 s in any conditions (data not shown), although the autolysis shown in Fig. 6 was for 2 min to permit comparison with other experiments. This is significantly faster than autolysis of 80k/\(Δ86\), as measured by the fall in \([\text{Ca}^{2+}]_{1.05}\), which in the presence of 0.18 M NaCl and 5 mg/ml casein required approximately 270 s for completion (Fig. 5a). It has been reported elsewhere that large subunit autolysis in m-calpain was extremely slow, but the measurements depended on antipeptide antibodies that may not have been sufficiently diagnostic of autolysis (22).

The delay in autolysis in the presence of casein (Fig. 5b) suggested that large subunit autolysis in m-calpain could also be at least to some extent an intermolecular reaction, just as for the small subunit. In keeping with this idea, it has recently been shown that \(μ\)-calpain could autolyze m-calpain in *vivo*, suggesting a cascade mechanism for its *vivo* activation of m-calpain (37). In that report, m-calpain activation was stated to be due to small subunit hydrolysis, and a fall in \([\text{Ca}^{2+}]_{1.05}\) of m-calpain was observed from 160 to 64 \(μM\) (measured in 10 mM HEPES, compare the value of 175 \(μM\) in 50 mM Tris in Fig. 1). We have shown, however, that small subunit autolysis does not affect \([\text{Ca}^{2+}]_{1.05}\) and in our view this fall in \([\text{Ca}^{2+}]_{1.05}\) was undoubtedly due to large subunit autolysis that could not be detected on gels.

**Activity of Intact m-Calpain**—It is evident that intact m-calpain must be active in the first steps of its own autolysis, and it has been suggested previously that intact m-calpain (at least with an intact large subunit) could hydrolyze substrates such as casein (17, 20, 24). This point has now been rigorously proven with the mutant L8F, A9F-80k/\(Δ86\), which hydrolyzes casein with the same specific activity and \([\text{Ca}^{2+}]_{1.05}\) as the other calpains described; on exposure to \(\text{Ca}^{2+}\) it loses activity (by inactivating cleavage of the large subunit) but does not undergo a fall in \([\text{Ca}^{2+}]_{1.05}\) and therefore does not undergo N-terminal autolysis (Fig. 8). The same conclusion could be drawn from the activity of 80k/\(Δ86\) in 0.4 M NaCl, where N-terminal autolysis also does not occur.

**Stability of Autolyzed m-Calpain**—It was reported previously that autolyzed m-calpain was less stable to dialysis at high ionic strength than at low ionic strength (23). This is reflected in our finding that \(Δ9–80k/\(Δ86\) formed by autolysis of various constructs was very poorly recovered from the MonoQ column, which requires elution at 0.4 M NaCl (Table III). We have made a related observation that the stability of the heterodimer containing an intact large subunit and an N-terminally truncated small subunit (80k/NHis-116) was also reduced at high ionic strength. Both these observations imply that salt links contribute to subunit binding. Autolyzed calpain is clearly not exposed to 0.4 M NaCl in *vivo*, but the loss of stability may still have in *vivo* implications that remain to be explored. Loss of \(Δ9–80k/\(Δ86\) in the presence of EDTA suggests that it is prone to processes such as subunit dissociation (3) and aggregation. The constructs M9–80k/\(Δ86\) and \(Δ9–80k/\(Δ86\) were designed as models of the natural autolysis product and were expressed at average levels as heterodimers. They were, however, inactive and stable to purification and therefore differ in some way from the natural autolysis product. They may be in some way improperly folded, or the free N-terminal lysine residue at position 10 may be especially important, but this point requires further work.

**Summary**—These studies have clarified a number of aspects

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**Table III**

| Form of calpain | Recovery of activity | Recovery after MonoQ%
|----------------|----------------------|-------------------|
| 80k-CHis/\(Δ86\) | 74% | 84% |
| 80k-CHis/\(Δ86\) | 73% | 50% |
| 80k/\(Δ86\) | 74% | 43% |
| L8F,A9F-80k-CHis/\(Δ86\) | 74% | 58% |
| NHHis₁₀-80k-CHis/\(Δ86\) | 60% | 20% |

*a* Percentage of activity remaining in the incubation mixture after brief autolysis, with respect to the control sample incubated in the same conditions but without \(\text{Ca}^{2+}\).

*b* Percentage of activity recovered after application of the autolysis incubation mixture to the MonoQ column, with respect to the control sample also applied to the MonoQ column.

*c* This incubation was carried out in 0.5 M NaCl without casein for 1 min.
of m-calpain biochemistry. For purified m-calpain in vitro, the pathways and rates of autolysis, and also the observed values of \([Ca^{2+}]_{0.5}\), are highly dependent on the experimental conditions. Autolysis of the small subunit in m-calpain in the presence of \(Ca^{2+}\) is rapid, is slightly retarded in the presence of casein, and does not affect either \([Ca^{2+}]_{0.5}\) or the stability of calpain. m-Calpain is fully active prior to autolysis of the large subunit, but in vitro at an approximately physiological ionic strength the intact large subunit has a short half-life in the presence of \(Ca^{2+}\), and it is assumed that the same is true in vivo. Autolysis of the large subunit at Ala\(^9\)-Lys\(^{10}\) may be a little slower than that of the small subunit and is also slightly retarded by the presence of casein, showing that at least part of the large subunit autolysis reaction is intermolecular. Although autolysis of both subunits can occur by intermolecular reaction, there are some grounds for believing that the reaction in vivo may be mainly intramolecular. Cleavage at Ala\(^9\)-Lys\(^{10}\) is responsible both for the fall in \([Ca^{2+}]_{0.5}\), and for the reduction in stability of the autolysis product. It is clear, however, that factors either in addition to, or other than, autolysis are involved in lowering \([Ca^{2+}]_{0.5}\) in vivo (1, 2), and calpain autolysis products cannot be detected in normal tissue extracts and must, therefore, be very rapidly cleared after activation (20). In our view, the physiological importance of autolysis is probably that it generates forms of active calpain with short half-lives, their instability, and their further auto-degradation, thus limiting the duration of calpain activity in vivo.

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