m-Calpain subunits remain associated in the presence of calcium

Previn Dutt[a], J. Simon C. Arthur[a], Dorothy E. Croall[b], John S. Elce[a,*]

[a] *Department of Biochemistry, Queen's University, and The Protein Engineering Network of Centres of Excellence, Kingston, Ont. K7L 3N6, Canada*
[b] *Department of Biochemistry, Microbiology and Molecular Biology, University of Maine, Orono, ME 04469-5735, USA*

Received 26 August 1998

Abstract The hypothesis that calpain subunits dissociate in the presence of Ca^{2+} has been tested by methods which avoid interference by Ca^{2+}-induced aggregation and large subunit autolysis. Inactive Cys^{105}Ser-*m*-calpain, bound either to Ni-NTA-agarose or to immobilized casein, after incubation with Ca^{2+}, could be recovered in high yield as a heterodimer. Natural bovine *m*-calpain, after irreversible inhibition with Z-LLY-CHN_2, also bound to immobilized casein and was eluted as a heterodimer. The Ca^{2+} requirements of calpain containing a small subunit with EF-hand mutations were higher, both before and after autolysis, than those of wild-type calpain. In mixtures of wild-type and mutant enzymes, subunit exchange did not occur in the presence of Ca^{2+}. The results demonstrate that the subunits in both natural and recombinant *m*-calpain, in the given experimental conditions, remain associated in the presence of Ca^{2+} both before and after autolysis.

Key words: *m*-Calpain; Autolysis; Subunit dissociation; EF-hand

1. Introduction

The two forms of calpain which can readily be isolated from vertebrate tissue are termed μ- and *m*-calpain. When carefully purified in the presence of EDTA, 2ME, and serine protease inhibitors, they are heterodimers of an 80-kDa large subunit and a 28-kDa small subunit. The large subunits of μ- and *m*-calpain share approximately 60% amino acid sequence identity, and the small subunits are identical. The enzymes are absolutely dependent on Ca^{2+}, and differ most obviously in their in vitro Ca^{2+} requirements for activity with a substrate such as casein: these values lie in the range of 5–50 μM Ca^{2+} for μ-calpain, and ~350 μM Ca^{2+} for *m*-calpain [1–4]. On exposure to Ca^{2+}, the enzymes undergo autolysis of both subunits: small subunit autolysis does not affect the Ca^{2+} requirement, but large subunit autolysis reduces the Ca^{2+} requirements of the enzymes, in *m*-calpain from 325 μM to 180 μM Ca^{2+}, and in μ-calpain from 5–50 μM to 1–5 μM Ca^{2+} [4,5]. Following this initial autolysis and in the continued presence of Ca^{2+}, further degradation occurs, mainly of the large subunit, which abolishes activity.

Regulation of calpain activity by Ca^{2+} has long been considered to involve binding of Ca^{2+} to EF-hands in the C-terminal domains of the large and small subunits, leading to a conformational alteration which activates the enzyme; this activation permits it to commence hydrolysis not only of itself (autolysis) but also of available substrates [1,2]. However, there have been several recent reports that the subunits in both μ- and *m*-calpain dissociate reversibly on addition of Ca^{2+}, and that the isolated large subunit possesses full calpain activity and a lower Ca^{2+} requirement [6–10]. In contrast, two other reports have argued against subunit dissociation in calpain [11,12]. It should be understood that these fundamentally contradictory reports arise at least in part from the technical difficulties of carrying out the relevant experiments in an unambiguous manner, since the addition of Ca^{2+} to calpain can cause aggregation, autolysis, and poor protein recoveries, any of which may make it difficult to interpret the results. We report here several results obtained with recombinant forms of *m*-calpain which minimize these difficulties and provide strong evidence against Ca^{2+}-induced subunit dissociation.

*Corresponding author. Fax: (1) (613) 545 2497.
E-mail: jse@post.queensu.ca

Abbreviations: 2ME, 2-mercaptoethanol; BSA, bovine serum albumin; C105S, the *m*-calpain large subunit active site Cys^{105}Ser mutation; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; His-tag, N-terminal His_{10}- and C-terminal -His_6 sequences; NTA, nitrilo-triacetic acid; Z-LLY-CHN_2, *N*-carbobenzoxy-L-leucyl-L-leucyl-L-tyrosyl-diazomethane

2. Methods

2.1. Recombinant calpains

Bacterial expression and purification of recombinant *m*-calpain, the inactive mutant C105S-*m*-calpain, and various forms of calpain with or without large subunit N-terminal His_{10}- and C-terminal -His_6 sequences (His-tags) have been described [4,13,14]. These calpains all contain the N-terminally truncated 21-kDa form of the calpain small subunit, which is very similar to the natural small subunit autolysis product [4]. We refer to this protein as 21k, although in terms of strict nomenclature it may be more correctly named Δ86-small subunit [4].

Glutamic acid to alanine mutations were made in 21k by means of site-directed mutagenesis at position 12 (−z) of the Ca^{2+} binding loop in each of the EF hands 1, 2, and 3 [15]. The resultant small subunit construct is described here as 21k-(EF1–3). The large subunit of *m*-calpain, with and without His-tags, was co-expressed with the wild-type 21k and with 21k-(EF1–3) in *E. coli* by standard methods, providing recombinant *m*-calpains in yields of 3–5 mg/l, which were fully active against casein in the standard assay at 5 mM Ca^{2+}.

2.2. Ca^{2+} treatment of inactive calpain bound to a Ni-NTA spin column

These experiments were performed at room temperature in a buffer composed of 50 mM Tris-HCl, pH 7.6, 0.2 M NaCl, 0.1% (v/v) Triton X-100 [6,7], 10 mM 2ME. Ni-NTA spin columns (Qiagen) were centrifuged at each step for 1 min at 3000 rpm, in accordance with the manufacturer's instructions, and the receiving tubes contained an aliquot of EDTA solution, such that the final net concentration of EDTA in the collected fluid was 2 mM.

C105S-*m*-calpain (C105S-*m*-80k-CHis_6/21k) (100 μg) was applied to duplicate Ni-NTA spin columns in 0.2 ml of buffer in the absence of Ca^{2+}. After washing in buffer without Ca^{2+}, the spin columns were incubated twice for 1–2 min with 0.2 ml of 5 mM Ca^{2+}, followed by centrifugation. Control samples were treated identically but with no Ca^{2+}. The columns were washed twice with 0.2 ml of buffer without Ca^{2+}, and then eluted twice with 0.2 ml of 0.4 M imidazole in the same buffer. The protein content of all samples was estimated by the Bradford assay [16], and appropriate samples analyzed by SDS-PAGE.

PII: S0014-5793(98)01167-3

2.3. Calcium-dependent casein-Sepharose affinity chromatography of inactive calpain

Casein and BSA were linked to CNBr-activated Sepharose according to the manufacturer's instructions. The resultant resins contained 3–4 mg of bound protein/ml of gel. The casein- or BSA-Sepharose matrix (1 ml) was mixed with 100 μg of C105S-*m*-calpain in 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 2 mM EDTA, 5 mM 2ME, at room temperature, and Ca^{2+} was then added to a final concentration of 5 mM. The mixtures were loaded into small columns, and after washing the columns in buffer with Ca^{2+}, bound protein was eluted with 5 mM EGTA in the same buffer. The protein in the various solutions was analyzed on SDS-PAGE as described above.

A similar experiment was carried out with *m*-calpain isolated from bovine heart as previously described [17]. This *m*-calpain, 250 μg in 1 ml of 50 mM MOPS, pH 7.5, 1 mM EDTA, 1 mM EGTA, 5 mM 2ME, was inactivated by pre-incubation with 100 μM Z-LLY-CHN_2 [18] for 15 min at 25°C and for an additional 15 min after the addition of Ca^{2+} to 5 mM. EGTA was added to terminate the reaction. Binding to immobilized casein and subsequent washing was carried out in 20 mM MOPS, pH 7.5, 0.1 M NaCl, 5 mM 2ME, in the presence of 5 mM Ca^{2+}; bound protein was eluted with 10 mM EGTA in the same buffer and the eluted protein was analyzed by SDS-PAGE and immunoblotting.

2.4. Calcium requirement for casein hydrolysis before and after large subunit autolysis

Prior to titration of their Ca^{2+} requirements, the enzymes were dialysed overnight at 4°C against 50 mM Tris-HCl (pH 7.6 at 20°C), 0.2 M NaCl, 2 mM EDTA, 10 mM 2ME, and adjusted to a concentration of approximately 250 U/ml (140 μg/ml). After dialysis, some samples were autolyzed by addition of Ca^{2+} to a final concentration of 5 mM, and incubation for 4–6 min at 25°C. The autolysis reaction was quenched by addition of an equal molar amount of EDTA. In control samples, EDTA was added before Ca^{2+}, so that autolysis did not occur but the final composition of the solution was the same. Ca^{2+} titrations were performed as previously described, but it should be noted that both the rates of autolysis of *m*-calpain, and the observed values of $[Ca^{2+}]_{0.5}$, are highly dependent on the pH and ionic strength of the reaction mixtures, which were therefore carefully standardized [4].

Important comparisons were made only between 2–3 enzymes dialyzed against the same buffer and titrated at the same time. The point of the experiments with recombinant calpains containing a small subunit with triple EF-hand mutations, is that these enzymes have greatly increased $[Ca^{2+}]_{0.5}$ values, so that the resulting differences in $[Ca^{2+}]_{0.5}$, before and after autolysis, are much greater than the possible experimental variations between assays at different times and in precise calcium concentrations.

2.5. Attempts to detect reversible subunit exchange by observation of changes in $[Ca^{2+}]_{0.5}$

$NHis_{10}$-*m*-80k-$CHis_6$/21k-(EF1–3) (100 μg, 1 nmol, active) and C105S-*m*-80k-$CHis_6$/21k (400 μg, 4 nmol, inactive) were separately incubated at 25°C with 5 mM Ca^{2+} for 5 min in 50 mM Tris-HCl, pH 7.6, 0.2 M NaCl, 10 mM 2ME; samples were taken for gel electrophoresis to demonstrate autolysis of the $NHis_{10}$- construct, the two enzymes were combined and thoroughly mixed, and autolysis was quenched by addition of EDTA. The Ca^{2+} requirement of the mixture of enzymes for casein hydrolysis was measured.

3. Results

3.1. Treatment of immobilized inactive calpain with Ca^{2+}

The inactive mutant calpain, C105S-*m*-80k-$CHis_6$/21k, was bound to Ni-NTA spin columns by means of the large subunit C-terminal His-tag. The spin columns were incubated with or without 5 mM Ca^{2+}, washed in buffer without Ca^{2+}, and bound protein was eluted with 0.4 M imidazole. The recovery of protein in each of the earlier washes and eluates was 2–3%, and in the imidazole eluate was 87%. These values were not affected by the presence or absence of Ca^{2+}. Traces of heterodimer, indicating leakage from the column, could be detected

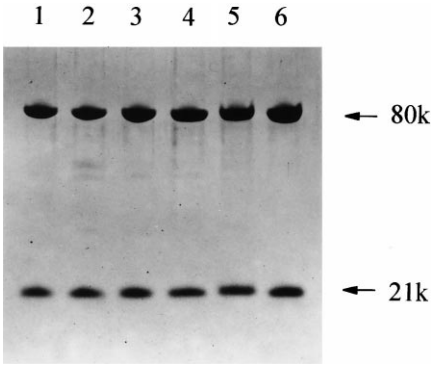

Fig. 1. Gel electrophoresis of immobilised calpain treated with Ca^{2+}. C105S-*m*-80k-$CHis_6$/21k was bound to N-NTA spin columns by means of the His-tag, or to casein-Sepharose in the presence of Ca^{2+}. The gel tracks contained: 1, 2: controls of enzyme bound to Ni-NTA, not treated with Ca^{2+}, and eluted with 0.4 M imidazole; 3, 4: enzyme bound to Ni-NTA, treated with 5 mM Ca^{2+}, and eluted with 0.4 M imidazole; 5: enzyme bound to casein-Sepharose in the presence of 5 mM Ca^{2+}, and eluted with 5 mM EGTA; 6: standard sample of the enzyme starting material.

both in the Ca^{2+}-treated and control eluates by means of immunoblot analysis but not by Coomassie staining, but the amounts did not differ between the Ca^{2+}-treated and control samples. The expected heterodimer of 80-kDa+21-kDa bands was observed in the imidazole eluates, and the bands did not differ between Ca^{2+}-treated and control samples, or from the starting material (Fig. 1). Similar experiments were done with *active* $NHis_{10}$-*m*-80k-$CHis_6$/21k calpain bound to Ni-NTA spin columns. Activity and protein assays and electrophoresis showed that the control samples (recovery 93%) were intact, and that the protein recovered from the Ca^{2+}-treated samples (yield of activity, 48%) was ~10% autolyzed. This indicates that most of the calpain lost in the samples exposed to Ca^{2+} had been autolyzed and further degraded while bound to Ni-NTA, and is consistent with the idea that autolyzed calpain is much less stable than intact enzyme [4].

The results of binding C105S-*m*-80k-$CHis_6$/21k to casein-Sepharose in the presence of Ca^{2+} were essentially identical to the Ni-NTA experiment. The protein was eluted from the column in high yield by EGTA, and was unchanged heterodimer (Fig. 1). BSA is not a substrate for calpain, and no protein was retained by the BSA-Sepharose column in the presence of Ca^{2+}. Bovine *m*-calpain which had been covalently inhibited by reaction with Z-LLY-CHN_2 also bound to immobilized casein in the presence of Ca^{2+} and was eluted as unchanged heterodimer (data not shown).

3.2. Ca^{2+} requirement of wild-type and mutant calpains before and after autolysis

Autolysis of the large subunit at Ala^9-Lys^{10}, which is responsible for the fall in Ca^{2+} requirement of *m*-calpain, was confirmed in the following experiments by gel electrophoresis, as previously described [4].

Introduction of the Glu to Ala mutation in EF-hands 1–3 of 21k raised the Ca^{2+} requirement of the resultant calpains. Fig. 2 compares the Ca^{2+}-titration curves of wild-type *m*-80k-$CHis_6$/21k and EF-hand-mutated *m*-80k-$CHis_6$/21k-(EF1–3) calpains. The values of $[Ca^{2+}]_{0.5}$ calculated from these curves were 351 μM and 956 μM, respectively.

Table 1, section A, lists the values of $[Ca^{2+}]_{0.5}$, measured

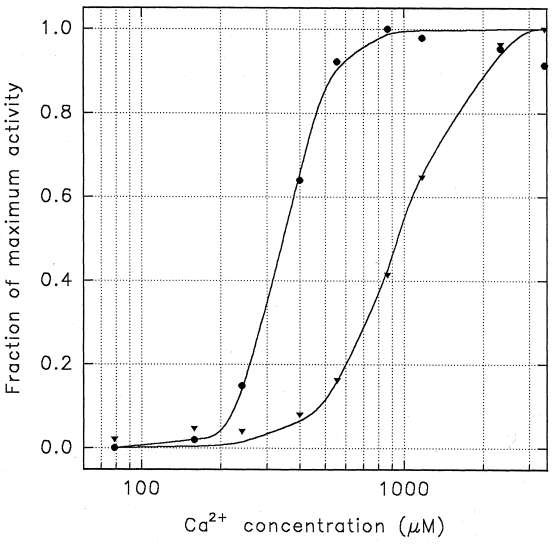

Fig. 2. Representative Ca^{2+} titration curves. These curves, obtained as previously described [4], show the effects of $[Ca^{2+}]$ on the activities of *m*-80k-$CHis_6$/21k (●) and *m*-80k-$CHis_6$/21k-(EF1–3) (▼). The derived values of $[Ca^{2+}]_{0.5}$ were 351 μM and 956 μM, respectively.

before and after autolysis, of $NHis_{10}$-*m*-80k-$CHis_6$/21k and of $NHis_{10}$-*m*-80k-$CHis_6$/21k-(EF1–3). It is important to note that the large subunit is identical in these two constructs. This experiment showed that $[Ca^{2+}]_{0.5}$ of Δ^9-*m*-80k-$CHis_6$/21k-(EF1–3), 361 μM, was significantly higher than that of Δ^9-*m*-80k-$CHis_6$/21k, 230 μM. Since the mutations in EF-hands 1–3 in the small subunit continued to influence the Ca^{2+} requirement of the whole enzyme, even after autolysis, the large and small subunits must have remained attached to each other. As discussed later, this conclusion would not be valid if subunit dissociation occurs reversibly, rapidly, and with high efficiency, even after autolysis.

The N-terminal His-tag allowed detection and quantification of autolysis on gels, and the C-terminal His-tag greatly improves expression of all these *m*-calpains in *E. coli*. However, to exclude the possibility that either of the large subunit His-tags might themselves prevent subunit dissociation, the Ca^{2+} titrations were repeated, before and after autolysis, on calpains with 21k and 21k-(EF1–3) but devoid of His-tags. The results (Table 1, section B) show that the Ca^{2+} requirements, and therefore the question of subunit dissociation, were not affected by His-tags.

3.3. Subunit exchange

If reversible subunit exchange occurs in a mixture of active $NHis_{10}$-*m*-80k-$CHis_6$/21k-(EF1–3) with a 4-fold molar excess of inactive C105S-*m*-80k-$CHis_6$/21k, following exposure to Ca^{2+} and autolysis, then the active enzyme after quenching with EDTA would be 80% Δ^9-*m*-80k-$CHis_6$/21k and 20% Δ^9-*m*-80k-$CHis_6$/21k-(EF1–3), which is predicted to have a Ca^{2+} requirement of ~220 μM. If subunit exchange does not occur, the only active component would be Δ^9-*m*-80k-$CHis_6$/21k-(EF1–3), predicted to have a Ca^{2+} requirement of ~350 μM. The results of this experiment are shown in Table 1, section C. The $[Ca^{2+}]_{0.5}$ values for the mixture were 1156 μM prior to autolysis and 324 μM following autolysis, corresponding to those expected for calpain containing the 21k-(EF1–3) mutated small subunit (Table 1, section A). These results establish that the 21k-(EF1–3) subunit was not replaced by the excess 21k subunit in the presence of Ca^{2+}, even after autolysis of the large subunit.

4. Discussion

4.1. Exposure of immobilized inactive calpain to Ca^{2+}

In the experiments with inactive calpains immobilized on Ni-NTA-spin columns or on casein-Sepharose, only traces of protein were eluted in buffer containing Ca^{2+}. Essentially all of the applied protein was recovered as intact heterodimer on elution with imidazole or EGTA, respectively. These results show that when inactive calpain was adsorbed to a solid matrix, so that both autolysis and aggregation were prevented, subunit dissociation did not occur in the presence of Ca^{2+}. The same result was obtained both for recombinant inactive calpains with His-tags, and for bovine *m*-calpain irreversibly inactivated with Z-LLY-CHN_2, showing that His-tags do not affect the experiment. The experiment was not affected by the means of attachment to the matrix: on Ni-NTA-agarose the binding is via the C-terminal His-tag, and is

Table 1
Effects of small subunit EF-hand mutations on Ca^{2+} requirement of *m*-calpain

	Large subunit	Small subunit	Autolysis	$[Ca^{2+}]_{0.5}$ (μM)
A	$NHis_{10}$-*m*-80k-$CHis_6$	21k	No	380
	Δ^9-*m*-80k-$CHis_6$	21k	Yes	230
	$NHis_{10}$-*m*-80k-$CHis_6$	21k-(EF1–3)	No	901
	Δ^9-*m*-80k-$CHis_6$	21k-(EF1–3)	Yes	361
B	*m*-80k	21k	No	422
	Δ^9-*m*-80k	21k	Yes	210
	m-80k	21k-(EF1–3)	No	1212
	Δ^9-*m*-80k	21k-(EF1–3)	Yes	309
C	1× $NHis_{10}$-*m*-80k-$CHis_6$/21k-(EF1–3) plus 4× C105S-*m*-80k-$CHis_6$/21k		No	1156
	Δ^9-*m*-80k-$CHis_6$/21k-(EF1–3) plus 4× C105S-*m*-80k-$CHis_6$/21k		Yes	324

The calpains consisted of wild-type large subunits with and without His-tags, and 21-kDa small subunits with and without Glu-Ala mutations in EF-hands 1, 2, and 3. Titrations were carried out before and after large subunit autolysis, which was caused by incubation with 5 mM Ca^{2+} for 4–6 min.

not Ca^{2+}-dependent; while on casein-Sepharose the binding is via the active site of the enzyme, and is Ca^{2+}-dependent. Casein-Sepharose has been used previously for isolation of active calpain, but in retrospect it is clear that some degradation of the enzyme could not be avoided [19]. Since in most of this work inactive calpains were used, these experiments by themselves do not exclude the possibility that subunit dissociation might be a consequence of large subunit autolysis. This question was addressed by means of Ca^{2+} titration assays.

4.2. Ca^{2+}-titration of calpain ± small subunit EF-hand mutations

The Ca^{2+} titration assays, with calpains containing both wild-type small subunits and EF-hand mutant small subunits, have demonstrated that subunit dissociation does not occur even after calpain autolysis. This is shown by the $[Ca^{2+}]_{0.5}$ value of Δ^9-*m*-80k-$CHis_6$/21k-(EF1–3), 361 μM, which was significantly higher than the value of 230 μM for the wild-type control Δ^9-*m*-80k-$CHis_6$/21k (Table 1, A). Since the mutated small subunit continued to influence the Ca^{2+} requirement of the large subunit, it must have remained associated with it. It has also been shown that the large subunit His-tags did not affect the observed $[Ca^{2+}]_{0.5}$ values.

In experiments involving brief exposure of active calpain to Ca^{2+} to cause autolysis, we were concerned that quenching of the autolysis reaction with EDTA, prior to Ca^{2+} titration, might promote re-association of subunits. Several titrations were carried out so that enzyme was taken directly from the autolysis reaction (at 5 mM Ca^{2+}) to the titration (where it was exposed immediately to 0.04–4 mM Ca^{2+}) without EDTA quench. This procedure did not, however, affect the observed $[Ca^{2+}]_{0.5}$ values (data not shown).

In evaluating the results of the autolysis and Ca^{2+}-titration experiments, we considered two possible cases which could invalidate our conclusions. In the first case, it may be postulated that the calpain subunits dissociate in the presence of Ca^{2+}, but do not significantly re-associate even on addition of EDTA. Then the $[Ca^{2+}]_{0.5}$ value of 361 μM observed after autolysis of $NHis_{10}$-*m*-80k-$CHis_6$/21k-(EF1–3), would be a property of the isolated autolysed wild-type large subunit, Δ^9-*m*-80k-$CHis_6$. However, $[Ca^{2+}]_{0.5}$ for autolyzed *m*-80k-$CHis_6$/21k, which in the case of irreversible subunit dissociation would also be isolated Δ^9-*m*-80k-$CHis_6$, has been shown to be ∼200 μM, but this value was not observed. The hypothesis of non-reversible subunit dissociation in *m*-calpain cannot therefore be correct.

In the second case, it may be postulated, as proposed elsewhere [6,7], that subunits dissociate in high $[Ca^{2+}]$ (for example during a prior autolysis incubation), but are able to reassociate rapidly in much lower $[Ca^{2+}]$ or on addition of EDTA. (It seems unlikely that 3–4 μg of dissociated calpain subunits could re-associate with high efficiency in 0.5 ml in the presence of 2 mg of casein, but this could not a priori be excluded.) In this case the $[Ca^{2+}]_{0.5}$ value of 361 μM observed after autolysis of $NHis_{10}$-*m*-80k-$CHis_6$/21k-(EF1–3) would actually represent $[Ca^{2+}]_{0.5}$ for re-dissociation of Δ^9-*m*-80k-$CHis_6$/21k-(EF1–3). To investigate this point, subunit exchange experiments were carried out with mixtures of $NHis_{10}$-*m*-80k-$CHis_6$/21k-(EF1–3) and 4 molar equivalents of C105S-*m*-80k-$CHis_6$/21k. On exposure to Ca^{2+} (accompanied by large subunit autolysis), subunit dissociation should mix the free 21k and 21k-(EF1–3) subunits in the soluble phase, and subsequent re-association would generate a mixture in which the major active component would be Δ^9-*m*-80k-$CHis_6$/21k. This would be expected to have $[Ca^{2+}]_{0.5}$ close to 200 μM, but this also was not observed, so that reversible subunit dissociation in *m*-calpain is not supported by the data presented here.

4.3. Conflicting reports concerning subunit dissociation

The results described here agree with earlier reports that the calpain subunits do not dissociate in the presence of Ca^{2+} [8,11,12,20]. However, there have been several reports of subunit dissociation in the presence of Ca^{2+} [6–10]. The reasons for this discrepancy are not clear, but one possible explanation lies in the fact that autolysis of the small subunit in natural calpains gives rise to heterogeneous products in the range ∼20–25 kDa which are very difficult to detect and quantify on the usual SDS/Tris-glycine gels. These problems were avoided in our experiments by working mainly with heterodimers containing the cloned 21k, which is not further autolyzed, and by using SDS/Tris-Tricine gels which are better suited for proteins of this size.

When autolysis is prevented by the C105S mutation, or minimized by irreversible inhibition with Z-LLY-CHN_2, calpain remains heterodimeric on exposure to Ca^{2+}. This was shown here with C105S-*m*-calpain bound to Ni-NTA-agarose and to casein, and again with Z-LLY-CH-bovine *m*-calpain bound to casein; but it had been shown earlier by the recovery of heterodimeric calpain from Z-LLY-CH-human μ-calpain bound in the presence of Ca^{2+} to a peptide derived from Ca^{2+}-ATPase [20], and to a peptide derived from calpastatin [8]. In the latter report, the 80k and 30k subunits were described as 'not present in equimolar amounts', which suggests that a small percentage of 30k autolysis to 20–25k fragments had occurred during treatment with inhibitor.

Reversible inhibitors probably do not prevent autolysis so efficiently, especially during column chromatography. It is also known that autolyzed calpains are stable for short periods in solution but give poor yields on column chromatography. It is therefore possible that the proteins described as isolated large subunits, obtained following Ca^{2+} treatment of active calpains in the presence of reversible inhibitors [6,8], were in fact heterodimers of autolyzed 80k+20–25k subunits, whose small subunit components were not easily detectable. These 'isolated large subunits' had a reduced $[Ca^{2+}]_{0.5}$, but this also is consistent with the suggestion that they were autolyzed heterodimers. Other results presented here of Ca^{2+}-titration of $NHis_{10}$-large subunit constructs and (EF1–3)-mutated small subunit constructs, have shown that the initial step of large subunit autolysis also does not lead to subunit dissociation.

We conclude that in these recombinant forms of *m*-calpain, both before and after large subunit autolysis, in the conditions of our experiments, subunit dissociation did not occur. Since the subunit association is non-covalent, the binding reaction must have a dissociation constant, K_{diss}, but its value must be extremely small.

Acknowledgements: This work has been supported by grants to J.S.E. from the Medical Research Council of Canada, the Protein Engineering Network of Centres of Excellence (PENCE), and the Faculty of Medicine, Queen's University; and by grants to D.E.C. from the National Science Foundation (USA) (MCB9319602 and NSF 97-23636). The collaboration was undertaken in part while D.E.C. was a sabbat-

ical visitor at Queen's University. We thank Ms Carol Hegadorn at Queen's University and Ms Deborah Reid at the University of Maine for outstanding technical assistance.

References

[1] Croall, D.E. and DeMartino, G.N. (1991) Physiol. Rev. 71, 813–847.
[2] Goll, D.E., Thompson, V.F., Taylor, R.G. and Zalewska, T. (1992) BioEssays 14, 549–556.
[3] Sorimachi, H., Ishiura, S. and Suzuki, K. (1997) Biochem. J. 328, 721–732.
[4] Elce, J.S., Hegadorn, C. and Arthur, J.C. (1997) J. Biol. Chem. 272, 11268–11275.
[5] Saido, T.C., Nagao, S., Shiramine, M., Tsukaguchi, M., Sorimachi, H., Murofushi, H., Tsuchiya, T., Ito, H. and Suzuki, K. (1992) J. Biochem. 111, 81–86.
[6] Yoshizawa, T., Sorimachi, H., Tomioka, S., Ishiura, S. and Suzuki, K. (1995) Biochem. Biophys. Res. Commun. 208, 376–383.
[7] Yoshizawa, T., Sorimachi, H., Tomioka, S., Ishiura, S. and Suzuki, K. (1995) FEBS Lett. 358, 101–103.
[8] Anagli, J., Vilei, E.M., Molinari, M., Calderara, S. and Carafoli, E. (1996) Eur. J. Biochem. 241, 948–954.
[9] Vilei, E.M., Calderara, S., Anagli, J., Berardi, S., Hitomi, K., Maki, M. and Carafoli, E. (1997) J. Biol. Chem. 272, 25802–25808.
[10] Michetti, M., Salamino, F., Minafra, R., Melloni, E. and Pontremoli, S. (1997) Biochem. J. 325, 721–726.
[11] Zhang, W. and Mellgren, R.L. (1996) Biochem. Biophys. Res. Commun. 227, 890–896.
[12] Elce, J.S., Davies, P.L., Hegadorn, C., Maurice, D.H. and Arthur, J.S.C. (1997) Biochem. J. 326, 31–38.
[13] Elce, J.S., Hegadorn, C., Gauthier, S., Vince, J.W. and Davies, P.L. (1995) Protein Eng. 8, 843–848.
[14] Arthur, J.S.C., Gauthier, S. and Elce, J.S. (1995) FEBS Lett. 368, 397–400.
[15] Blanchard, H., Grochulski, P., Li, Y., Arthur, J.S., Davies, P.L., Elce, J.S. and Cygler, M. (1997) Nat. Struct. Biol. 4, 532–538.
[16] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[17] Croall, D.E. and DeMartino, G.N. (1984) Biochim. Biophys. Acta 788, 348–355.
[18] Crawford, C., Mason, R.W., Wikstrom, P. and Shaw, E. (1988) Biochem. J. 253, 751–758.
[19] DeMartino, G.M. and Croall, D.E. (1983) Biochemistry 22, 6287–6291.
[20] Molinari, M., Maki, M. and Carafoli, E. (1995) J. Biol. Chem. 270, 14576–14581.