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Dissociation of m-Calpain Subunits Occurs after Autolysis of the N-Terminus of the Catalytic Subunit, and Is Not Required for Activation[1]

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Calpain is a heterodimeric, intracellular Ca^{2+}-dependent, "bio-modulator" that alters the properties of substrates through site-specific proteolysis. It has been proposed that calpains are activated by autolysis of the N-terminus of the large subunit and/or its dissociation into the subunits. It is, however, unclear whether the dissociation into subunits is required for the expression of protease activity and/or for *in vivo* function. Recently, the crystal structure of m-calpain in the absence of Ca^{2+} has been resolved. The 3D structure clearly shows that the N-terminus of the m-calpain large subunit (mCL) makes contact with the 30K subunit, suggesting that autolysis of the N-terminus of mCL changes the interaction of both subunits. To examine the relationship between autolysis, dissociation, and activation, we made and analysed a series of N-terminal mutants of mCL that mimic the autolysed forms or have substituted amino acid residue(s) interacting with 30K. As a result, the mutant m-calpains, which are incapable of autolysis, did not dissociate into subunits, whereas those lacking the N-terminal 19 residues (Δ19), but not those lacking only nine residues (Δ9), dissociated into subunits even in the absence of Ca^{2+}. Moreover, both Δ9 and Δ19 mutants showed an equivalent reduced Ca^{2+} requirement for protease activity. These results indicate that autolysis is necessary for the dissociation of the m-calpain subunits, and that the dissociation occurs after, but is not necessary for, activation.

Key words: activation, autolysis, calpain, dissociation, EF-hand.

Calpain is a cytosolic cysteine protease that requires Ca^{2+} for activity, and plays important roles in various biological phenomena regulated by Ca^{2+} in the cell (*1–6*). The physiological function of calpain is so important that if the regulation of activity is impaired, it can cause diseases such as neurofibromatosis type 2 (*7, 8*), Alzheimer's disease (*9, 10*), and muscular dystrophies (*11, 12*). In order to understand the molecular mechanisms of these diseases, it is essential to clarify the method of activation and activity regulation of calpain.

Two forms, μ- and m-calpain, are ubiquitously expressed in animal tissues and have been well studied. Both form heterodimers consisting of a homologous but distinct catalytic 80-kDa subunit (μCL and mCL) and a common regulatory 30-kDa subunit (30K). The *in vitro* protease activity of calpain requires Ca^{2+} concentrations much higher than those that occur under physiological conditions; μ- and m-calpains require 5–50 μM, and 0.2–1 mM Ca^{2+}, respectively. Thus, a mechanism to decrease the Ca^{2+} requirement of calpain is necessary for expression of the activity *in vivo*. It has previously been reported that the Ca^{2+} requirement is reduced by autolysis of the N-terminus of the catalytic subunit (*13–16*), in the presence of lipid (*17–20*), by addition of an activator protein (*21, 22*), or by dissociation of the subunits (*23–28*). However, some studies have shown that m-calpain:C105S (in which the active site Cys-105 is converted to Ser resulting in no protease activity) does not dissociate into subunits even in the presence of Ca^{2+} (*29, 30*). It is still unclear whether dissociation into subunits is essential for activation and/or the *in vivo* functions of calpain.

Recently, the crystal structure of m-calpain in the absence of Ca^{2+} has been elucidated (*31, 32*). To our surprise, it showed that the N-terminus of mCL (domain I) is buried in the hydrophobic pocket of the 5-EF-hand domain of 30K. Specific amino acid residues form salt bridges, as described in the "RESULTS." Thus, it has been suggested that autolysis of the N-terminus of mCL plays an important role in the activation and/or dissociation of calpain. To

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Abbreviations. mCL, m-calpain large subunit; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; Suc-LLVY-MCA, succinyl-Leu-Leu-Val-Tyr-methylcoumarine amide.

clarify the relationship between autolysis, activation, and dissociation, we made several recombinant m-calpain mutants and examined their properties.

MATERIALS AND METHODS

Construction of Mutants That Mimic the Autolysed Form of m-Calpain—PCR was carried out using the human m-calpain large subunit (mCL) or mCL:C105S in pTV119N (*33*) as a template, and the following oligonucleotides as primers: Sense primers: 5′-AAGGCCTGGACCGCAGCATGAAGGACCGGGAGGCGGCCGA-3′ (for mCL:Δ9), and 5′-AAGGCTTGGACCGCAGCATGTCCCACGAGAGGGCCATCAAG-3′ (for mCL:Δ19), containing an *Stu*I site at the 5′ end. Antisense primer, 5′-GCTCTAGATCAAAGTACTGAGAAACAGAGCCA-3′, containing an *Xba*I site at the 5′ end. PCR products were digested with *Stu*I and *Xba*I, and ligated between *Stu*I and *Xba*I sites of pFastBacI (GIBCO BRL; MD, USA) to make pFastBacI-mCL C105S:Δ9 or -mCL:C105S:Δ19.

Construction of Mutants with Altered Residues That Interact with 30K—Mutations in the residues that interact with 30K were produced by ligation-free PCR-mediated mutagenesis using the human m-calpain large subunit (mCL) or mCL:C105S cDNA in pTV119N as a template, and the following and complementary oligonucleotides as primers: For mCL:K7L and mCL:C105S K7L, 5′-GCATCGCGGCCGGCCTGGCGAAGGAC-3′ For mCL: R12L and mCL:C105S:R12L, 5′-TGGCGAAGGACCTCGAGGCGGCCGA-3′. mCL:C105S:K7L:R12L was similarly produced using the oligonucleotides for mCL:R12L and mCL:C105S:K7L generated above. The mutated sequences were verified by full-length DNA sequencing. The mutated cDNAs were digested with *Nco*I, treated with the Klenow fragment of DNA polymerase I, digested with *Hin*dIII and subsequently inserted between the *Stu*I and *Hin*dIII sites of pFastBacI.

Construction of N-Terminally His-Tagged 30K (30K:N-His)—Human 30K cDNA was digested with *Hin*cII and *Hin*dIII, ligated between pET-16b, digested with *Bam*HI, treated with S1 Nuclease, and digested with *Hin*dIII. Human 30K in pET-16b was digested with *Xba*I and *Hin*dIII, and ligated between the *Xba*I and *Hin*dIII sites of pFastBacI.

Expression by the Sf-9/Baculo Virus System—Recombinant viruses were prepared according to the manufacturer's protocol for "Bac-to-Bac Baculovirus Expression Systems" (GIBCO BRL). Cell culture and expression of recombinant m-calpain and its mutants were performed as described previously (*33*).

Analysis of Dissociation into Subunits Using a Ni^{2+}-Affinity Column—Cells expressing each of various mutant mCLs and 30K:N-His simultaneously were harvested by centrifugation (300 ×*g*), and the pellets were suspended in buffer A (20 mM Tris/Cl [pH 7.5], 150 mM NaCl, 0.01 mM EGTA, 5 mM 2-mercaptoethanol, and 10 mM imidazole) supplemented with 0.1 mM leupeptin. The cells were lysed by sonication, ultracentrifuged at 100,000 ×*g* for 30 min, and loaded onto a 1 ml Ni^{2+}-NTA agarose column (QIAGEN; CA, USA). The column was washed with three column volumes (3× CV) of buffer A supplemented with 60 mM imidazole, followed by 2× CV of buffer A supplemented with 1 mM Ca^{2+}, and then 2× CV of buffer A supplemented with 300 mM imidazole (flow rate: 0.5 ml/min). All experiments were performed at 8°C. The eluted fractions were examined for the presence of mCL protein.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting—Each fraction from the above Ni^{2+}-affinity column was mixed with SDS-PAGE loading buffer (62 mM Tris-HCl [pH 6.8], 143 mM 2-mercaptoethanol, 2% (w/w) SDS, 0.005% (w/w) bromophenol blue, and 10% (v/v) glycerol), boiled at 100°C for 5 min, and electrophoresed in a 10% (w/v) polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue G250, or transferred to a PVDF membrane (Millipore; MA, USA) and immunostained using polyclonal anti-m-calpain antiserum (*34*).

Analysis of Ca^{2+} Requirement for Protease Activity—Protease activities of the mutants were assayed using succinyl-Leu-Leu-Val-Tyr-methylcoumarine amide (Suc-LLVY-MCA) purchased from Peptide Institute (Osaka), as described previously (*35*).

RESULTS

Dissociation into Subunits of Full-Length m-Calpain—To confirm that m-calpain dissociates in the presence of Ca^{2+} (*23*), recombinant m-calpain composed of the wild-type full-length m-calpain large subunit (mCL:WT) and N-terminally His-tagged 30K (30K:N-His) was used (denoted as mCL·WT+30K:N-His hereafter). The protein expressed in Sf-9 cells was directly applied to a Ni^{2+}-affinity column, and eluted with buffer containing 1 mM Ca^{2+} followed by 300 mM imidazole, as described in "MATERIALS AND METHODS." When mCL:WT+30K:N-His was applied, more than 90% of the total mCL was detected in the elution fraction with 1 mM Ca^{2+} (Fig. 1, lane 1) The band corresponding to 30K was detected mostly in the elution fraction containing 300 mM imidazole. These results are consistent with the previous observation (*23*); however, the possibility cannot be dismissed that the N-terminal part of 30K in-cluding the His-tag was autolysed to elute the whole heterodimer molecule

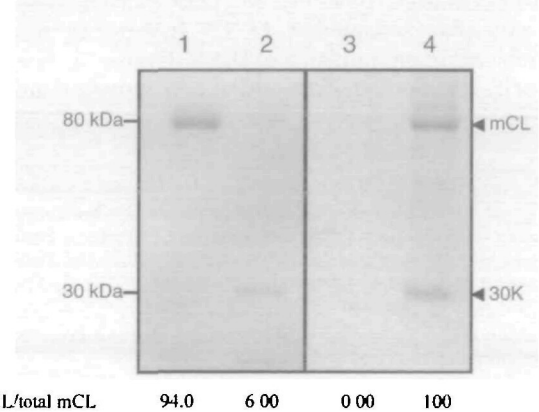


Fig 1 **Dissociation of full-length m-calpain into the subunits.** Sf-9 cell lysates that co-expressed mCL (lanes 1 and 2) or mCL·C105S (lanes 3 and 4) and 30K:N-His were applied to a Ni^{2+}-affinity column, washed, and eluted with 1 mM Ca^{2+} (lanes 1 and 3) followed by 300 mM imidazole (lanes 2 and 4) as described in "MATERIALS AND METHODS." Fractions were analysed by SDS-PAGE, and the gels were stained with CBB Relative amounts of mCL show the ratio of mCL in each lane to the total amount of mCL in all lanes. The mCL band intensities were quantified by densitometry.

of mCL and truncated 30K without dissociation. Next, inactive recombinant m-calpain composed of mCL.C105S (active-site Cys105 substituted with Ser) and 30K:N-His was examined. In this case, mCL:C105S did not elute with 1 mM Ca^{2+} (Fig. 1, lane 3), but co-eluted with 30K in the imidazole fraction (lane 4). This result reinforces previous reports that m-calpain:C105S does not dissociate into subunits (*29, 30*) These results with WT and C105S strongly suggest that m-calpain does not dissociate in the presence of Ca^{2+}, and that the observed apparent dissociation is an autolytic artifact, as described above. From these results, another possible explanation is that the dissociation of m-calpain requires autolysis of the N-terminus of mCL. N-terminal sequence analysis indicated that more than 50% of the mCL in the fraction eluted with 1 mM Ca^{2+} was autolysed (data not shown), although N-terminal autolysis of mCL is not detectable by SDS-PAGE analysis (*36*). It is possible that the N-terminal autolysis of mCL allows m-calpain to dissociate. Thus, we investigated this possibility.

Interaction between 30K FE-Hand Motifs and the N-Terminus of mCL—The crystal structure of m-calpain in the absence of Ca^{2+} (*31, 32*) revealed that the N-terminus of mCL is located in the hydrophobic pocket of 30K (Fig. 2a). Moreover, Lys7 and Arg12 of mCL make salt-bridges with Asp154 of the EF-2 and Glu260 of the EF-5 of 30K, respectively, as judged from the distances between these molecules. The EF-2 binds Ca^{2+}, whereas the EF-5 does not (*37*). Because autolysis of the N-terminus of mCL occurs between Ala9 and Lys10 and between Gly19 and Ser20 (Ref. *36*, Fig. 2b), we considered the possibility that the N-terminal-cleaved forms of mCL may affect the dissociation of the subunits. Therefore, we made mutants mimicking the autolysed forms of mCL (mCL:Δ9, and mCL:Δ19), with a non-charged amino acid (Leu) instead of Lys7 and/or Arg12 (mCL:K7L, mCL:R12L, and mCL:K7L:R12L), and their inactive further mutants (mCL:C105S:Δ9, *etc*), and examined their functional properties.

Heterodimer Stability of Autolysed Forms of m-Calpain—When the inactive truncation-mutant mCL:C105S:Δ9+30K:N-His was applied to the Ni^{2+}-affinity column, as in the case of the full length inactive m-calpain, mCL:C105S+30K:N-His, it did not dissociate; that is, mCL:C105S:Δ9 was not eluted with Ca^{2+} and most of the protein was eluted in the imidazole fraction (Fig. 3, lanes 1 to 3). Next, when mCL:C105S:Δ19 was used instead of Δ9, 50% of mCL:C105S:Δ19 was eluted in the flow-through fraction (lane 4). The 30K subunit was detected predominantly in the imidazole fraction (lane 6). mCL·C105S (data not shown) and mCL:C105S:Δ9 (lane 1) were almost undetectable in the flow-through fraction. Therefore, these results suggest that the interaction between mCL:C105S:Δ19 and 30K.N-His is weaker than in the wild-type protein, probably because the absence of two salt-bridges between the N-terminus of mCL and 30K makes them unable to form a stable heterodimer. This possibility was supported by their behavior in column chromatography. When mCL:C105S:Δ19+30K:N-His was purified by sequential column chromatography using DEAE-Toyopearl, gel-filtration, and Mono

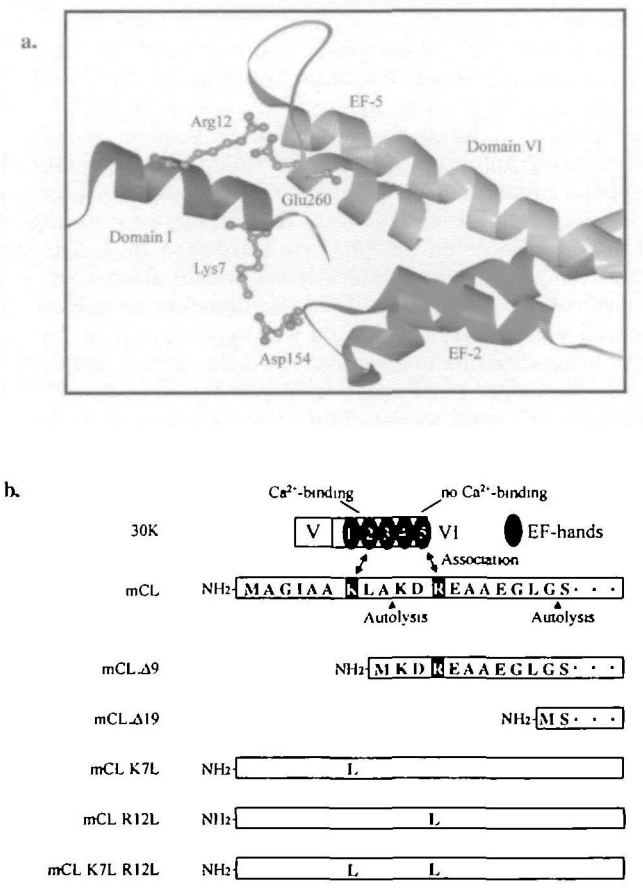
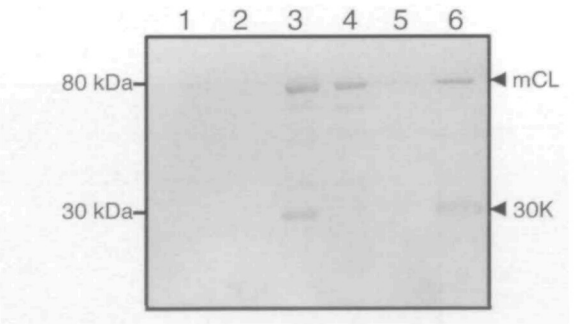


Fig 2 **Interactions between the N-terminus of mCL and 30K.** a Schematic ribbon 3D structure of domains I and VI of human m-calpain in the absence of Ca^{2+} (*32*). Lys7 and Arg12 in the N-terminus of mCL (blue) interact with Asp154 in the loop of the EF-2 (red) and Glu260 in the α-helix of the EF-5 (orange) of 30K, respectively The distances strongly suggest salt bridges form in these interactions b: Schematic structures of the mutant mCLs used in this study. mCL·Δ9 and :Δ19 start with Lys10 and Ser20, respectively, after the initiation Met at the N-termini. mCL:K7L, :R12L, and K7L:R12L have Leu instead of Lys7, Arg12, or both, respectively. The rest of the sequence is identical to that of the wild type



mCL/total mCL (x100) 0 00 0 00 100 49 3 5 20 45.5

Fig 3. **Heterodimer stability and dissociation of the autolysed forms of m-calpain.** Sf-9 cell lysates that co-expressed mCL·C105S Δ9 (lanes 1–3) or mCL C105S:Δ19 (lanes 4–6) and 30K.N-His were applied to a Ni^{2+}-affinity column, washed with buffer, and eluted with 1 mM Ca^{2+} (lanes 2 and 5) followed by 300 mM imidazole (lanes 3 and 6) as described in "MATERIALS AND METHODS." The flow-through was also collected (lanes 1 and 4) Fractions were analysed similarly as in Fig. 1 by SDS-PAGE, and detected by immunoblotting using anti m-calpain antiserum. Relative amounts of mCL were determined as in Fig. 1.

Q, the dissociated subunits could be distinguished (data not shown). This was not observed in the case of mCL:C105S +30K:N-His and mCL:C105S:Δ9+30K:N-His (data not shown).

Dissociation into Subunits of Mutants at Amino Acid Residues That Interact with 30K—If the two salt-bridges are essential to maintain the heterodimer formation of m-calpain, then mCL:K7L should have the same properties as mCL:Δ9, as both mutants commonly lack the interaction between Lys7 of mCL and Asp154 of the EF-2 of 30K. Similarly, mCL:K7L:R12L must have the same properties as mCL:Δ19, because both mutants commonly lack the interactions between Arg12 of mCL and Glu260 of 30K in addition to that between Lys7 and Asp154. As expected, when mCL:C105S:K7L+30K:N-His was applied to the Ni^{2+}-affinity column, as with mCL:C105S+30K:N-His and mCL:C105S:Δ9+30K:N-His, it did not dissociate—it was not eluted in either the Ca^{2+} or the flow-through fraction, and was eluted in the imidazole fraction (Fig. 4, lanes 1–3). As in the case of mCL:C105S:Δ19+30K:N-His, the large subunit of mCL:C105S:K7L:R12L+30K:N-His was eluted in the flow-through fraction (Fig. 4, lane 7)

When mCL:C105S:R12L+30K:N-His was used, about a half the amount of mCL:R12L was eluted with Ca^{2+}, but not in the flow-through fraction (Fig. 4, lane 4). This means that mCL:C105S:R12L+30K:N-His dissociates into subunits in a Ca^{2+}-dependent manner. This mutant retains the interaction between Lys7 of mCL and Asp154 in the loop region of the EF-2 of 30K. Since the EF-2 binds Ca^{2+}, the Lys7–Asp154 salt bridge must be destroyed upon Ca^{2+}-binding, as predicted previously (*32*). Thus, these results can be interpreted as follows: mCL:C105S:R12L+30K:N-His dissociates into subunits upon the addition of Ca^{2+} because it loses the salt bridge between Lys7 and the EF-2 due to Ca^{2+}-binding, and has a mutated interaction site between Arg12 and the EF-5.

Of the other mutants, mCL:K7L retains the interaction between Arg12 of mCL and the EF-5 of 30K, and the EF-5 does not bind Ca^{2+}. Thus, mCL:C105S:K7L+30K:N-His did not dissociate even in the presence of Ca^{2+}. In conclusion, two interactions, Lys7–Asp154 and Arg12–Glu260, are important for the heterodimer formation of the large and small subunits of m-calpain. Either interaction is enough to maintain the association, but m-calpain dissociates into subunits when both interactions are lost.

The Effects of Autolysis of the mCL N-Terminus on the Ca^{2+} Requirement for Protease Activity—It has been suggested that the autolysis of the first nine amino acid residues of the N-terminus of mCL decreases the Ca^{2+} requirement for protease activity (*15, 38*). It is, however, unclear whether or not autolysis of the first 19 amino acid residues of mCL further lowers the Ca^{2+} requirement. As shown above, m-calpain dissociates into subunits after autolysis of the 19 N-terminal residues, but not the first nine residues, of mCL. Our previous study showed that the Ca^{2+} requirement for protease activity of mCL alone without 30K is identical to that of autolysed m-calpain (*23*). Thus, it must be clarified whether dissociation into subunits further lowers the Ca^{2+} requirement for protease activity after autolysis of the N-terminus of mCL, or whether autolysis alone is sufficient to decrease the Ca^{2+} requirement.

To address the above points, we examined the Ca^{2+} requirement of the active mutants used in the above experiments (mCL:Δ9, :Δ19, :K7L, .R12L, and :K7L:R12L+30K:N-His). All mutants were purified by sequential column chromatography or Ni^{2+}-affinity and MonoQ anion-exchange columns. Their specific activities just after purification were almost equivalent to that of wild-type m-calpain (Table I). However, the protease activity of mCL:Δ19 and mCL:K7L:R12L was completely lost within about a week of purification, showing that these mutants are unstable compared with other mutants or wild-type m-calpain. In the following experiments, we measured the activity using proteins just after purification (within 1 h). As shown in Fig 5a, the Ca^{2+} requirement of mCL:Δ9 was lower than that of wild type m-calpain, consistent with the previous reports (*15, 38*). The Ca^{2+} requirement of mCL: Δ19 was identical to that of mCL:Δ9, indicating that further autolysis of Δ9 to Δ19 does not result in an additional decrease in the Ca^{2+} requirement for protease activity. The Ca^{2+} requirements of mCL:K7L and mCL:K7L:R12L were also lower than that of wild-type m-calpain to the same extent as mCL:Δ9 and mCL:Δ19 (Fig. 5b). On the other hand, the Ca^{2+} requirement of mCL:R12L was identical to that of wild type m-calpain (Fig. 5b). In summary, the Ca^{2+} requirement for the protease activity of mutants that lack the interaction between Lys7 of mCL and the EF-2 of 30K, that is,

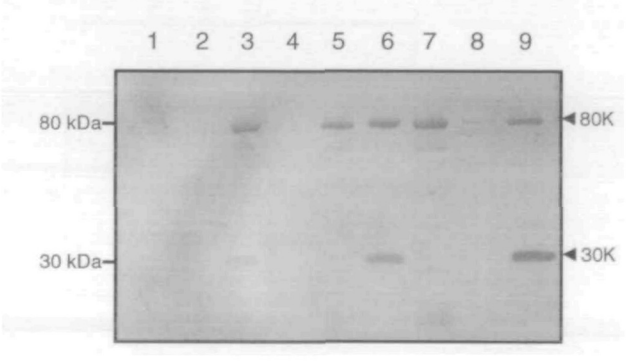


Fig. 4. **Dissociation of the Lys7 and Arg12 mutants.** Sf-9 cell lysates that co-expressed mCL:C105S K7L (lanes 1–3), mCL:C105S R12L (lanes 4–6), or mCL:C105S.K7L.R12L (lanes 7–9) and 30K.N-His were applied to a Ni^{2+}-affinity column and eluted with 1 mM Ca^{2+} (lanes 2, 5, and 8) followed by 300 mM imidazole (lanes 3, 6, and 9) as described in "MATERIALS AND METHODS." The flow-through was also collected (lanes 1, 4, and 7) Fractions were analysed as in Fig. 3. Relative amounts of mCL were determined as in Fig. 1.

TABLE I **Specific activities of the purified mutants.** The yield of protein is given as the total amount of protein purified from 2.0 × 10^8 Sf-9 cells. The yield of activity is given as the total units of activity from 2 0 × 10^8 Sf-9 cells, measured just after purification

	Yield of protein (μg)	Yield of activity (units)	Specific activity	
			Just after purification (units/mg)	A week after purification (units/mg)
WT	1,200	485	404	403
Δ9	701	270	385	380
Δ19	150	52 5	350	0 00
K7L	599	227	379	381
R12L	649	252	388	382
K7L:R12L	130	45.8	353	0.00

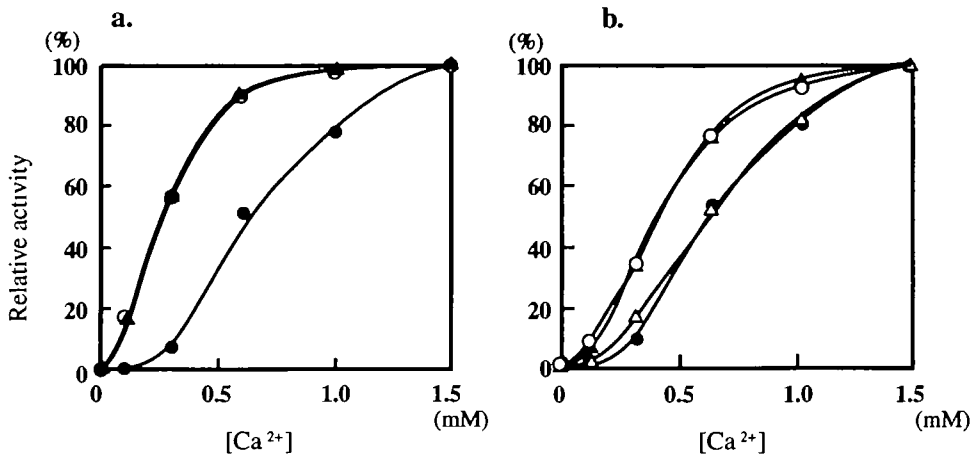


Fig 5 **Ca^{2+} requirement for protease activity of the N-terminal mutants.** Protease activity was measured with Suc-Leu-Leu-Val-Tyr-MCA as a substrate as described in "MATERIALS AND METHODS" a Ca^{2+} requirement for protease activity of the mutants mimicking the autolysed forms [mCL Δ9+30K.N-His (▲), and mCL Δ19+30K.N-His (○)] compared with that of wild type m-calpain (●). b Ca^{2+} requirement for protease activity of the Lys7 and/or Arg12 mutants [mCL K7L (▲), mCL R12L (△), and mCL K7L R12L (○)] compared with that of wild type m-calpain (●)

TABLE II. **Properties of m-calpain mutants used in this study.**

	Salt bridge		30K binding		Ca^{2+} requirement	Stability
	Lys7–Asp154	Arg12–Glu260	$-Ca^{2+}$	$+Ca^{2+a}$		
WT	+	+	+	+	high	high
Δ9	–	+	+	+	low	high
Δ19	–	–	+–	+–	low	low
K7L	–	+	+	+	low	high
R12L	+	–	+	+–	high	high
K7L R12L	–	–	+–	+–	low	low

[a]C105S inactive mutants were used

mCL:Δ9, :Δ19, :K7L, and K7L·R12L, were lower than that of wild type m-calpain, whereas the Ca^{2+} requirement of mCL:R12L, which dissociates into subunits in a Ca^{2+}-dependent manner, was identical to that of wild type m-calpain (Table II). These results indicate that the dissociation of m-calpain into subunits does not decrease the Ca^{2+} requirement for protease activity (*i.e.*, the activation of m-calpain does not require dissociation into subunits).

DISCUSSION

Since the model of m-calpain activation by subunit dissociation was proposed (*23, 24*), some researchers have objected because of the observation that m-calpain:C105S and m-calpain:C105S:Δ9 do not dissociate into subunits (*29, 30*). In this report, it was clarified that two salt-bridge interactions between mCL and 30K, Lys7–Asp154 and Arg12–Glu260, are essential for the heterodimer formation of m-calpain, and that m-calpain is susceptible to dissociation into subunits when both of these interactions are lost, for example, by autolysis of 19 amino acid residues of the N-terminus of mCL, or by mutations of K7L and R12L. These results, therefore, consistently explain the apparently discrepant results about the dissociation of m-calpain into subunits, that is, Δ19 autolysis is necessary for dissociation. On the other hand, the autolysis of 30K probably does not affect dissociation, because it was reported that mCL:C105S and truncated 30K (20K) do not dissociate into subunits even in the presence of Ca^{2+} (*29, 30*). In this study, mCL:K7L:R12L and mCL:Δ19 were very unstable compared with other mutants or the wild-type enzyme, and the possibility that those mutations abolished the m-calpain 3D structure cannot be eliminated. However, since mCL:K7L and mCL.R12L were stable and the activity was indistinguishable from that of wild-type m-calpain, mCL:K7L·R12L is considered to have the proper structure. As previously reported, 30K also functions as a stabilizer of calpain (*24*) and mCL:K7L:R12L and :Δ19 tend to dissociate 30K. This is probably the reason for their instability. The previously reported result that wild-type m-calpain dissociates into subunits when protease inhibitors such as leupeptin or E64 are present in the gel filtration buffer (*23*) can be explained as follows. Since these inhibitors cannot completely suppress autolysis, especially N-terminal autolysis of the large subunit, and since free inhibitors become separated from m-calpain during gel filtration, N-terminal autolysis of mCL might have occurred during the experiments, and this was followed by dissociation. Retrospectively, autolysed fragments of mCL can be seen in the fraction containing the dissociated mCL (Ref. *23*, Fig. 1b). On the other hand, in the previous study in which both subunits of either m- or μ-calpain were co-immunoprecipitated in the presence of Ca^{2+} (*39*), autolysis of the 19 amino acid residues at the N-terminus of mCL might not have occurred. The crystal structure of m-calpain in the absence of Ca^{2+} shows that the protease activity of m-calpain is latent in the absence of Ca^{2+} because the protease domain is divided into two subdomains separately containing the catalytic Cys105 and His262 residues, preventing the formation of the catalytic center (*31, 32*). In the presence of Ca^{2+}, however, the two subdomains should fuse to form a functional catalytic active-site like as in other cysteine proteases. In this study, we showed that the Ca^{2+} requirement for the protease activity of mutants that lack the interaction between Lys7 of mCL and the EF-2 of 30K is lower than that of wild-type m-calpain (see Fig. 5 and Table II). These results strongly

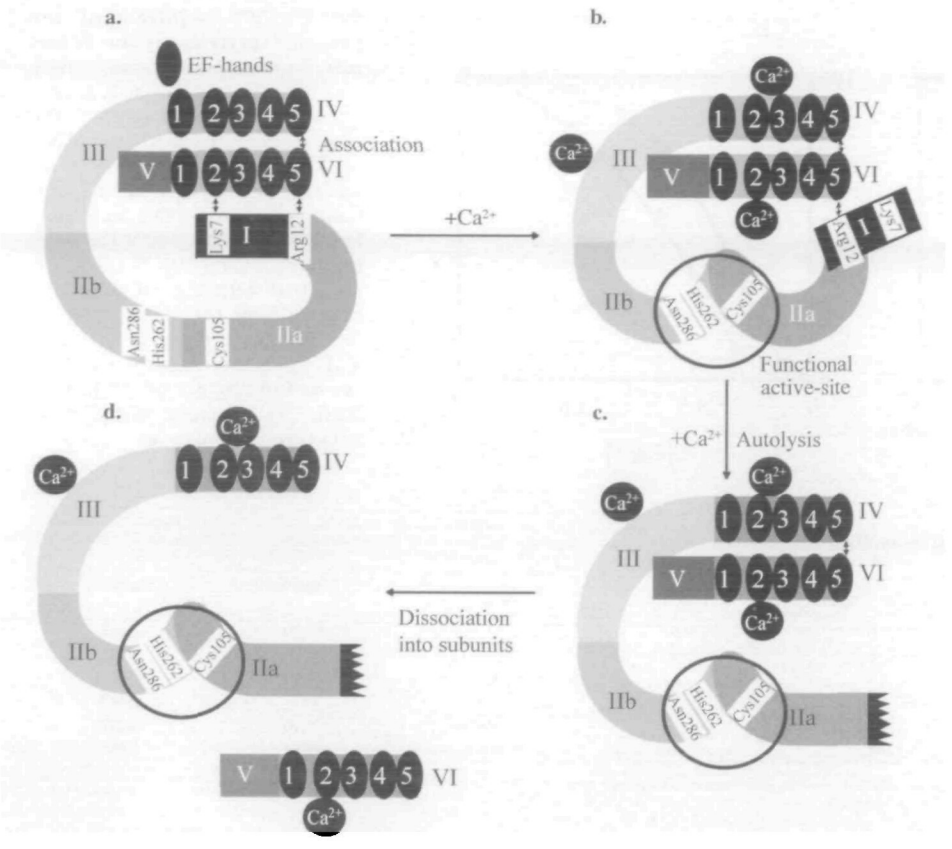


Fig 6 **A model of the structural changes in the activation of m-calpain by Ca^{2+}.** In the absence of Ca^{2+}, the catalytic center has not formed because of the physical distance between Cys105 and His262 of mCL (a) The catalytic active-site is formed by dynamic conformational changes upon Ca^{2+} binding Among these changes, destruction of the interaction between Lys7 of mCL and the EF-2 of 30K in the presence of Ca^{2+} plays a major role in the release of protease subdomain IIa (b) After formation of the functional active-site, autolysis of the first 19 amino acid residues of the N-terminus of mCL occurs (c), leading to dissociation into subunits (d)

suggest that the salt-bridge between Lys7 and EF-2 anchors the Cys105-containing protease subdomain (IIa) to 30K and inhibits the formation of a functional active-site in the absence of Ca^{2+}. Therefore, it is very probable that destroying the interaction between Lys7 and the EF-2 of 30K by Ca^{2+} binding to the EF-2 releases subdomain IIa to allow the formation of a functional active-site (Fig. 6, a to b). Since the N-terminus of mCL is located in the hydrophobic pocket of 30K, the salt-bridge between Lys7 and the EF-2 of 30K must be protected from exposure to ions except for the specific binding of Ca^{2+}. Since the mutants that lose the interaction between Lys7 of mCL and the EF-2 of 30K do not require Ca^{2+} binding to the EF-2 of 30K to form the active-site, it is reasonable that the Ca^{2+} requirement for the protease activity of these mutants is lower than that of wild type m-calpain Although it has long been discussed whether or not the activation of calpain requires N-terminal autolysis of the calpain large subunit, autolysis is not necessarily required for the activation of m-calpain. After the formation of the functional active-site, autolysis of the N-terminus of mCL takes place followed by subunit dissociation (Fig. 6, c to d).

In this report, we show that the dissociation of m-calpain into subunits is not required for the activation of m-calpain, at least *in vitro*, although we cannot completely eliminate the possibility that m-calpain becomes activated after dissociation *in vivo*. Then, what are the physiological functions of dissociation? Many calmodulin binding proteins have been reported to be calpain substrates (*40*), and some substrates, such as cytokine receptor and presenilin, actually bind to the 5-EF-hand domain of calpain (*41, 42*). Thus, the 5-EF-hand domain is considered to be very important for substrate recognition. Calpain loses one of the two 5-EF-hand domains upon dissociation into subunits, which may alter its substrate specificity. Cytokine receptor γ chain has been reported to bind to the 5-EF-hand domain of 30K, suggesting that dissociation of calpain changes the susceptibility of this substrate (*41*). A model has been proposed in which calpain translocates to the membrane, is anchored by domain III of mCL (*43*) and/or with domain V of 30K (*44*), and is then activated (*19, 20*). After calpain dissociates into subunits upon activation at the membrane, it is tempting to speculate that only the catalytic subunit returns to the cytosol, and/or that the dissociated 30K independently functions as a Ca^{2+}-binding protein, not as a protease These possibilities are now under examination in our laboratory.

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