Autolysis of Calpain Large Subunit Inducing Irreversible Dissociation of Stoichiometric Heterodimer of Calpain

Hiroshi Kitagaki,1,2 Shigeo Tomioka,1 Toshio Yoshizawa,1 Hiroyuki Sorimachi,1,3 Takaomi C. Saito,4 Shoichi Ishiura,1,5 and Koichi Suzuki1

1Department of Molecular Biology, Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan
2Office of Brewing Technology, Osaka Regional Taxation Bureau, 1-5-63, Otemae, Chuo-ku, Osaka City, Osaka 540-0008, Japan
3Department of Applied Biological Chemistry, and Department of Applied Biological Engineering, Graduate School of Agricultural and Life Science, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan
4Laboratory for Proteinase Neuroscience, RIKEN Brain Science Institute, 2-1, Hirosawa, Wako, Saitama 351-0198, Japan
5Department of Life Science, Graduate School of Arts and Science, University of Tokyo, Meguro-ku, Tokyo 153-8902, Japan

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Calpain, a calcium dependent cysteine protease, consists of a catalytic large subunit and a regulatory small subunit. Two models have been proposed to explain calpain activation: an autolysis model and a dissociation model. In the autolysis model, the autolyzed form is the active species, which is sensitized to Ca2+. In the dissociation model, dissociated large subunit is the active species. We have reported that the Ca2+ concentration regulates reversible dissociation of subunits. We found further that in chicken μ-m-calpain autolysis of the large subunit induces irreversible dissociation from the small subunit as well as activation. So we could propose a new mechanism for activation of the calpain by combining our findings. Our model insists that autolysed large subunit remains dissociated from the small subunit even after the removal of Ca2+ to keep it sensitized to Ca2+. This model could be expanded to other calpains and give a new perspective on calpain activation.

Key words: calpain; activation; autolysis; dissociation of subunits; autolysis-induced irreversible dissociation model

Calpain is a calcium dependent cysteine protease consisting of a catalytic large subunit and a regulatory small subunit. The large subunit contains a cysteine protease domain similar in sequence to other cysteine proteases such as papain and cathepsins B, H, and L, and is thus regarded as the catalytic subunit. The small subunit is believed to be the regulatory subunit but its precise mechanism for regulation of large subunit activity is obscure.1-3

Calpain and its superfamily proteins are reported to be responsible for a wide variety of biological phenomena such as human limb-girdle muscular dystrophy type2A,6 alkaline adaptation and sporulation in Saccharomyces cerevisiae7 and Aspergillus nidulans,8 and the sex determination cascade during early development in nematodes.7

Since activation of calpain requires a higher Ca2+ concentration than the physiological Ca2+ concentration in the cytosol, several models have been proposed for the activation mechanism of calpain.1-3

It was first proposed that an autolysed form is the activated form of calpain (autolysis model), because the Ca2+ sensitivity of calpain increases significantly upon autocatalytic modification of the N-terminal regions of both subunits in accordance with an intracellular influx of Ca2+.9 This autolysed form has been observed also in cells after treatment with calcium ionophores.8,10 However, the precise mechanism of how autolysis raises Ca2+ sensitivity of calpain has

To whom correspondence should be addressed. Hiroshi Kitagaki, Office of Brewing Technology, Osaka Regional Taxation Bureau, 1-5-63, Otemae, Chuo-ku, Osaka City, Osaka 540-0008, Japan. Tel: +81-66-941-5331(2368); Fax: +81-66-941-9926; E-mail: hks-ktgk@ja2.so-net.ne.jp

Abbreviations: calpain A, calpain incubated at 0°C in 10 mM Tris-HCl, pH 7.5, containing 1 mM 2-mercaptoethanol, 100 mM NaCl, 1.5 mM CaCl2, and 0.2 mM leupeptin (a calpain inhibitor) and 5 mM EDTA added after 1 min; calpain B, calpain incubated at 0°C in 10 mM Tris-HCl, pH 7.5, containing 1 mM 2-mercaptoethanol, 100 mM NaCl, and 1.5 mM CaCl2, and 5 mM EDTA added after 1 min; calpain C, calpain incubated at 0°C in 10 mM Tris-HCl, pH 7.5, containing 1 mM 2-mercaptoethanol, 100 mM NaCl, and 1.5 mM CaCl2, and 5 mM EDTA added after 30 min
not been elucidated.

Independently of the autolysis model, we have insisted that the dissociated large subunit is the active species of calpain (dissociation model), because dissociated large subunit possesses proteolytic activity indistinguishable from intact calpain, and Ca\(^{2+}\) sensitivity indistinguishable from autolyzed calpain, and proposed the Ca\(^{2+}\) concentration for the mechanism that induces reversible dissociation into subunits (Ca\(^{2+}\)-induced dissociation model). However, Zhang and Mellgren criticized the idea that Ca\(^{2+}\) concentration induces dissociation into subunits by insisting that calpain large subunit remains associated with small subunit in the presence of Ca\(^{2+}\).

By studying the interaction between the large and small subunits of the prepared calpain forms with two-dimensional electrophoresis, we propose a model that reconciles the autolysis model and dissociation model in this report, which gives a new perspective on calpain activation.

**Materials and Methods**

**Substrates and Preparation of calpain.** Casein was purchased from Merck. Guanidine hydrochloride (GuHCl) and polyethylene glycol 4,000 (PEG) were from Wako Pure Chemicals. Calpain was purified from chicken skeletal muscle as previously described. Purified calpain samples were dialyzed before use against 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, 1 mM 2-mercaptopoethanol, and 100 mM NaCl.

**Preparation of several forms of calpain.** Calpain was incubated at 0°C for 1 min in 10 mM Tris-HCl, pH 7.5, containing 1 mM 2-mercaptopoethanol, 100 mM NaCl, 1.5 mM CaCl\(_2\), and 0.2 mM leupeptin (a calpain inhibitor). Otherwise, calpain was incubated at 0°C in 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM 2-mercaptopoethanol, 100 mM NaCl, and 1.5 mM CaCl\(_2\) or at 10°C in 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM 2-mercaptopoethanol, 100 mM NaCl, and 1.5 mM CaCl\(_2\), the reaction solution was withdrawn after different intervals as described in the text and 5 mM EDTA was added to stop the reaction.

**Native and SDS-PAGE analysis.** The first electrophoresis (native PAGE) was done without SDS in 7.5% acrylamide gel. The lane was cut and equilibrated with SDS-PAGE loading buffer (62 mM Tris-HCl buffer, pH 6.8, 143 mM 2-mercaptopoethanol, 2% SDS, 0.005% bromophenol blue, 10% glycerol) at 37°C for 30 min, and the second electrophoresis (SDS-PAGE) was done with SDS in 12.5% acrylamide gel.

**Assay of calpain.** Calpain was incubated at 30°C with casein (3 mg/ml) in 200 μl of 100 mM Tris-HCl buffer, pH 7.5, containing 5 mM 2-mercaptopoethanol. Reaction was started by adding a final 2 mM CaCl\(_2\), and the mixture was incubated for 30 min. The reaction was stopped by the addition of 200 μl of 10% (w/v) trichloroacetic acid. After cooling for 20 min at 0°C, the sample was centrifuged at 8000 × g for 5 min and the A\(_{250}\) of the supernatant was recorded.

**Amino acid sequencing.** Chicken μ/m-calpain was autolyzed as described in the text. Peptides were separated by SDS-polyacrylamide gel electrophoresis followed by being botted onto a sheet of PVDF membrane, and were stained with Coomassie Brilliant Blue. Peptide bands were cut and amino acid sequences were analyzed in a Protein sequencer 477A-120A (Applied Biosystems).

**Results**

**Preparation of autolyzed forms of calpain**

We first prepared several autolyzed forms of calpain by adding sufficient CaCl\(_2\) to the reaction mixture and analyzed the samples by SDS-PAGE (Fig. 1). The intact calpain preparation gives an 80-kDa band corresponding to the intact large subunit but shows a double band of about 30-kDa for the small subunit. This is due to N-terminal truncation of the small subunit presumably by other proteases during purification (Tomoka, unpublished data). To prepare a calpain form having intact large subunit and autolyzed small subunit, and that having autolyzed large subunit and autolyzed small subunit, which require different autolysis conditions, calpain was incubated at 0°C and 10°C, respectively, and withdrawn after different intervals.

The calpain sample withdrawn after 1 and 3 min of autolysis at 0°C gave a clear band of 80 kDa for the intact large subunit and multiple bands of 20–30 kDa for partially autolyzed small subunit (Fig. 1A). Withdrawn after 30 min of autolysis at 10°C, the sample gave two clear bands of 78 kDa for the autolyzed large subunit and 18 kDa for the autolyzed small subunit (Fig. 1B). The calpain sample autolyzed for 1 min at 0°C was used as the calpain form having intact large subunit and partially autolyzed small subunit (calpain B), and that autolyzed for 30 min at 10°C was used as the calpain form having autolyzed large subunit and autolyzed small subunit (calpain C). Since in chicken μ/m-calpain autolysis of the small subunit precedes that of the large subunit, the calpain form having autolyzed large subunit and intact small subunit could not be analyzed in this approach. In order to prepare a calpain form having intact large subunit and intact small subunit under the same conditions as with calpains B and C, intact calpain was incubated at 0°C in 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM 2-mercaptopoethanol, 100 mM NaCl, 1.5 mM CaCl\(_2\), and 0.2 mM...
The Ca$^{2+}$-sensitivity of intact and autolyzed forms of calpain

The Ca$^{2+}$ sensitivities of autolyzed forms of calpain were measured in comparison with intact calpain and the results are summarized in Fig. 2. Intact calpain and calpain B show a $K_a$ (Ca$^{2+}$ concentration required for 50% maximal activity) of 0.3 mM, while the value for calpain C is 0.1 mM. Autolysis of the large subunit significantly increased calcium sensitivity, confirming the results reported previously. Calpain A, B, and C showed indistinguishable enzyme activities if they were compared in the specific casemolytic activities for large subunits.

Native PAGE analysis of intact and autolyzed forms of calpain

Calpain A, B, and C were analyzed by native PAGE to discover the association of calpain heterodimers. One major band was observed in calpain A and B (Fig. 3, lane A and B). This major band is considered to be the stoichiometric heterodimer of calpain. To the contrary in calpain C, proteins migrated with a smeared appearance with long tailing, and no distinct bands were observed. This indicated that molecular states of calpain changed significantly on autolysis of the large subunit. The smeared appearance would be attributed to the changes in association manner of proteins, since only limited changes were observed in the subunit molecules, especially between calpain B and C (Fig. 1). Long tailing suggested large but not stoichiometric aggregation of the proteins. No distinct protein bands were observed in the corresponding position to the major one in calpain A and B, and this suggested that the calpain C no longer had the stoichiometric heterodimer structure with a large and a small subunit.

Two-dimensional electrophoresis of intact and autolyzed calpain

To investigate the changes observed in Fig. 3 more precisely, we analyzed proteins of calpain A, B, and C in two-dimensional electrophoresis. Proteins of the calpain preparations were separated as in Fig. 3, and then the proteins were run on SDS-PAGE. Results are given in Fig. 4.

In the case of calpain A, the spot the molecular mass of which corresponds to intact large subunit (filled arrow) migrated together with the spots with molecular masses corresponding to intact small subunit (blank arrows). Large subunit indicated by filled
arrows were separated into two spots, both of which were accompanied by corresponding small subunits with different molecular masses (Fig. 4A). The results indicates that the intact calpain is a stoichiometric heterodimer with a large and a small subunit as established previously.

In the case of calpain B, a single spot the molecular mass of which corresponded to intact large subunit (filled arrow) migrated together with multiple spots the molecular mass of which corresponded to partially autolysed small subunit (blank arrows) (Fig. 4B). This result indicates that partial autolysis of the small subunit did not impair association of subunits and calpain B retained the stoichiometric heterodimer structure with the same Ca\(^{2+}\) requirement as intact calpain for activity (Fig. 2).

In contrast in the case of calpain C, two long and narrow spots of protein were prominent (Fig. 4C). The one with larger molecular mass located in the left half of the gel and was identified as the autolysed large subunit by the molecular mass and amino acid sequencing in a separate experiment. The spot was denser in a similar position (filled arrow) to those of intact large subunits in Fig. 4A and B, and projected and thinned out to the left, namely to the top of the first gel. The shape of the spot could explain the upper half tailing of proteins in Fig. 3C, and suggested that a considerable part of the 78 kDa protein was in non-stoichiometric aggregates. In addition to the tailing in the first-dimensional electrophoresis, the autolysed large subunit showed tailing even in the second dimension. The large spot at the filled arrow in Fig. 4C was apparent because of the tailing in the second dimension and the large amount of protein put on to detect the small subunit clearly. No substantial difference was found between Fig. 3C and Fig. 4C.

**Fig. 3.** Native Gel Analysis of Intact and Autolysed Forms of Calpain.

Samples (10 μg) were analyzed on 7.5% acrylamide gel without SDS. Protein was stained with Coomassie Brilliant Blue. Lane A: calpain A, Lane B: calpain B, Lane C: calpain C. Calpain A, B, and C were prepared as described in the text.

**Fig. 4.** Two-dimensional Electrophoresis of Intact and Autolysed Forms of Calpain.

Samples (10 μg) were separated by two-dimensional electrophoresis. The first electrophoresis was done on 7.5% acrylamide gel without SDS. The second electrophoresis was done on 12.5% acrylamide gel with 1.5% SDS. (A): calpain A, The protein was stained with Coomassie Brilliant Blue. Filled arrow indicates large subunit and blank arrows indicate small subunit. (B): calpain B, The protein was stained with Coomassie Brilliant Blue. Filled arrow indicates large subunit and blank arrows indicate small subunit. (C): calpain C, The protein was stained with Coomassie Brilliant Blue. Filled arrow indicates spot of the autolysed large subunit. The spot was denser in a similar location to those of intact large subunit as seen in panel A and B. The spot appeared as a large one because of tailing also in the second dimension, and thus it was seen apparently different form that in Fig. 3C. Blank arrow indicates the most distant position of the spot of small subunit from large subunit. (D): calpain C, The gel was analyzed by immunoblot using rabbit immune serum against human m-calpain as the first antibody. Only the portion of the blot containing the spot spreading from the blank arrow in (C) is shown. Calpain A, B, and C were prepared as described in the text.

Immunoblot analyses with an antiserum against human m-calpain identified all the part of spot including tailing consisted of calpain. The tailing in the second-dimensional electrophoresis was reproducible in the autolysed large subunit by the method described in Materials and Methods. The tailing is considered to be a specific property of the autolysed large subunit, since no such tailing could be observed in intact large subunits in Fig. 4A and B analyzed in the same methods. This would be explained by insufficient
denaturation of the autolyzed large subunit in equilibration with SDS sample buffer at a low temperature (37°C). No such tailing was observed if the sample was prepared by heating at 95°C. The other with lower molecular mass was in the right half of the gel and consisted of a long and narrow one with almost uniform thickness and a separate spot (blank arrow). The shape of the long spot could explain the lower half of smear proteins in Fig. 3C. To identify the protein, we analyzed first the spot by immunoblot with antiserum against human m-calpain and showed that all the parts of the spot were derivatives of the calpain (Fig. 4D). Then we analyzed the amino acid sequence of the 18-kDa band of calpain C in a separate experiment. The result indicated that the band consisted of two different peptides. The major one was that of the autolytic product of small subunit (VDPNEEERVRQFRRRTQLA) and amounted to about 80% of the total peptides. The other was a peptide of the large subunit that began with the 529th isoleucine (ITEDDIEGFKNMFQQLAGE) and amounted to about 20%. No other peptides were detected. We conclude from these data that most of the spot was the autolyzed small subunit. The shape of the spot suggested also that almost all the 18-kDa protein was in non-stoichiometric aggregates. Some round spots were also seen in Fig. 4C, which would be the fragments of large subunit autolyzed further than the 78-kDa intermediate. It is important in Fig. 4C to note that most of the spots of the large and small subunit did not overlap each other, and most of the projection of both spots were not accompanied by other proteins. The observation indicates that autolysed forms of large and small subunit were dissociated from each other, and individually formed aggregates by themselves. Even in the overlapped part, no clear packed spots as in calpain A and B were seen and the smear spot of the small subunit was far less dense than the corresponding spot in calpain A and B, which suggested that interaction of both subunits were occasional if any. Since calpain C showed caseinolytic activity indistinguishable from those of calpain A and B, calpain C would express the activity in the homologously aggregated form.

Discussion

We showed in this report that chicken μ/m-calpain significantly changed its molecular structure on autolysis of both subunits. Autolyzed large subunit no longer associated with autolyzed small subunit, the inhibitory regulatory subunit, even in the absence of Ca\(^{2+}\), and kept sensitized to Ca\(^{2+}\). Figure 4C indicated that spots of autolyzed large and small subunits migrated mostly separated in the native gel, and the observation ruled out the possibility of stoichiometric reassociation of the large and small subunits after autolysis even in the absence of Ca\(^{2+}\). The data suggested irreversible dissociation of stoichiometric heterodimer of calpain subunits. Shapes of the spots that projected and thinned out to the left from their original positions suggested that each subunit formed homogenous but non-stoichiometric aggregates. It is likely that the dissociated large subunits are in association-dissociation equilibrium among themselves and so are the small subunits. The homogenous aggregate of large subunit would show the calpain activity with higher Ca\(^{2+}\) sensitivity.\(^{11,12}\) Although small parts of both subunits overlapped each other, the parts did not form distinct round spots and thus interaction of both subunit would be occasional or non-stoichiometric if any. Total enzyme activity of the calpain C could not be explained by the remnant stoichiometric heterodimer species.

Based on the data reported in this paper and recent studies,\(^{13-17}\) the following model that reconciles the autolysis model and dissociation model can be proposed for the calpain. The model is as follows: Ca\(^{2+}\) influx into cytoplasm activates calpain via dissociation or/and autolysis of subunits, although dissociation is now controversial. Removal of Ca\(^{2+}\) from cytosol leaves two active calpain species, one with intact large and small subunits and the other with autolyzed large and small subunits. The former reassociates into the inactivated form, but the latter remains dissociated in the absence of Ca\(^{2+}\), keeping the sensitized state to Ca\(^{2+}\). The latter calpain is considered to be “irreversibly activated form” of calpain. The irreversibly activated form of calpain is considered to form non-stoichiometric aggregates in our experiments. This could be interpreted by elevation of adhesive properties of both subunits. It is tempting to assume that the autolysis increases the transfer of calpain to biological membranes or organelles to perform proper physiological functions such as maintenance of excitation of nerves in vivo. It is established that mammalian μ-calpain is localized onto membranes on activation in vivo.\(^{29}\)

We here designate this model the “autolysis-induced irreversible dissociation model”. We summarized the model and compared to the previous ones in Fig. 5. The autolysis model simply hypothesizes the autolytic form as the activated form, and thus calpain is a single-use enzyme. This contradicts the fact that calpain is a long-life enzyme.\(^{29}\) The Ca\(^{2+}\)-induced dissociation model is that Ca\(^{2+}\) causes dissociation of calpain and dissociated large subunit is the active enzyme. This model requires another explanation for the fact that autolyzed large and small subunit could be reproducibly observed after activation of calpain. The autolysis-induced irreversible dissociation model hypothesizes the autolyzed form as an irreversibly potentiated form that continued to retain activity for a certain duration. This model does not contradict autolysis and dissociation models, but complements them. At the same time, this model also
In the autolysis model, intracellular influx of Ca\(^{2+}\) causes calpain to autolyze and calpain is sensitized to Ca\(^{2+}\) after the removal of Ca\(^{2+}\) from the cytosol.

In the Ca\(^{2+}\)-induced dissociation model, intracellular influx of Ca\(^{2+}\) causes dissociation of calpain subunits and dissociated large subunit hydrolyzes the substrate. The large subunit showed the same Ca\(^{2+}\) sensitivity as the autolysed calpain, as measured by separating from small subunit through gel filtration and dialyzing against EDTA solution. After the removal of Ca\(^{2+}\) from the cytosol, the large subunit reassociates with the small subunit and returns to the inactivated form.

In the autolysis-induced irreversible dissociation model, intracellular influx of Ca\(^{2+}\) causes autolysis of calpain subunits. Dissociation of subunits by influx of Ca\(^{2+}\) is controversial at present. After removal of Ca\(^{2+}\) from the cytosol, autolyzed large subunit remains irreversibly dissociated from small subunit and sensitized to Ca\(^{2+}\).

Further studies, including the study to expand our model to other calpain species and to detect the autolysed intermediates in living cells are required to confirm our model. Elucidation of the activation mechanism of calpain will affect many fields such as medicine, development of drugs and brewing industry.

References


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