Dissociation and Aggregation of Calpain in the Presence of Calcium*

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Calpain is a heterodimeric Ca\(^{2+}\)-dependent cysteine protease consisting of a large (80 kDa) catalytic subunit and a small (28 kDa) regulatory subunit. The effects of Ca\(^{2+}\) on the enzyme include activation, aggregation, and autolysis. They may also include subunit dissociation, which has been the subject of some debate. Using the inactive C10S-S-80k/21k form of calpain to eliminate autolysis, we have studied its disassociation and aggregation in the presence of Ca\(^{2+}\) and the inhibition of its aggregation by means of crystallization, light scattering, and sedimentation. Aggregation, as assessed by light scattering, depended on the ionic strength and pH of the buffer, on the Ca\(^{2+}\) concentration, and on the presence or absence of calpastatin. At low ionic strength, calpain aggregated rapidly in the presence of Ca\(^{2+}\), but this was fully reversible by EDTA. With Ca\(^{2+}\) in 0.2 M NaCl, no aggregation was visible but ultracentrifugation showed that a mixture of soluble high molecular weight complexes was present. Calpastatin prevented aggregation, leading instead to the formation of a calpastatin-calpain complex. Crystallization in the presence of Ca\(^{2+}\) gave rise to crystals mixed with an amorphous precipitate. The crystals contained only the small subunit, thereby demonstrating subunit dissociation, and the precipitate was highly enriched in the large subunit. Reversible dissociation in the presence of Ca\(^{2+}\) was also unequivocally demonstrated by the exchange of slightly different small subunits between \(\mu\)-calpain and m-calpain. We conclude that subunit dissociation is a dynamic process and is not complete in most buffer conditions unless driven by factors such as crystal formation or autolysis of active enzymes. Exposure of the hydrophobic dimerization surface following subunit dissociation may be the main factor responsible for Ca\(^{2+}\)-induced aggregation of calpain. It is likely that dissociation serves as an early step in calpain activation by releasing the constraints upon protease domain I.

The classical \(\mu\)- and m-calpains are cytosolic Ca\(^{2+}\)-dependent cysteine proteases that are ubiquitously expressed and differ in their sensitivity to Ca\(^{2+}\). They consist of an isoform-specific catalytic ~80-kDa subunit (from the genes \(\text{capn}1\) and \(\text{capn}2\), respectively) and a common regulatory ~28-kDa subunit (\(\text{capn}4\)). Several other calpain-related genes are now known, but within this report, the word calpain refers only to the \(\mu\)- and m-calpains. Whereas the exact physiological roles of calpains remain to be defined, many studies suggest that they have important cellular roles. They have been implicated in several important cellular functions, including signal transduction, apoptosis, cell cycle regulation, and cytoskeletal reorganization (1–4). Unlike many other cysteine proteases, calpains tend to cleave substrates at interdomain boundaries, thereby serving to modulate the function of these substrates rather than simply digesting them (5). Several probable substrates have been identified both in vitro and in vivo including p53, protein kinase C, spectrin, Ca\(^{2+}\)-ATPase, talin, and fibronectin (4–8).

Both \(\mu\)- and m-calpain are absolutely dependent on Ca\(^{2+}\) for hydrolysis of their substrates (9–11). The initial effects of Ca\(^{2+}\) binding to calpain include a conformational change that is essential to assemble the active site and some limited autolysis of both subunits. Further results of Ca\(^{2+}\) binding include aggregation of the whole enzyme or continued autolysis and degradation. The recent structure determination of m-calpain (12, 13) has provided new insights into the structural basis of calpain activation by Ca\(^{2+}\). In the absence of Ca\(^{2+}\), the catalytic triad is not assembled so that the protease is inactive. Several structural features have been identified that maintain the active site in an inactive conformation. These involve on one side contacts between the large subunit N-terminal peptide of domain I and domain VI of the small subunit and on the other side contacts between domains II and III of the large subunit. Some of the later interactions have been clarified by mutational analysis (14), but the functional implications of the binding of the N-terminal peptide to domain VI in relation to enzyme activation, autolysis, dissociation, and aggregation are not well characterized.

Although there is a clear difference in the Ca\(^{2+}\) requirement for activation (250–350 \(\mu\)M for m-calpain and 10–50 \(\mu\)M for \(\mu\)-calpain), the basis for this difference remains elusive. However, the activation of both m- and \(\mu\)-calpain appears to be very similar, and it was proposed that one function of Ca\(^{2+}\) was to cause the dissociation of the calpain subunits, a dissociation that may be reversible before autolysis but is promoted and presumably irreversible following autolysis (15–17). In two other reports, however, subunit dissociation was not detected (18, 19). The inconsistent results stemmed from the complication of Ca\(^{2+}\)-induced aggregation of calpain, and the difficulty in designing unambiguous experiments. In the presence of heavy precipitation, further studies of the calpain activation process will become extremely difficult. Consequently, several important aspects of the process of calpain activation and subsequent aggregation remain poorly understood.

In our attempts to crystallize either wild-type or inactive m-calpain (C10S-S-80k/21k) in the presence of Ca\(^{2+}\), crystals were formed under certain conditions that contained only the

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small subunit together with an amorphous aggregate enriched in the large subunit. We therefore used light scattering and analytical ultracentrifugation to search for conditions in which Ca\(^{2+}\)-induced aggregation and possibly dissociation could be avoided. Aggregation of calpain has frequently been noted, but the factors involved have not been systematically studied (15, 16, 19, 20). Subunit dissociation was also studied by the above methods and also by an improved subunit exchange experiment. The results confirmed unequivocally the occurrence of subunit dissociation in the presence of Ca\(^{2+}\) and provided some new insight into the nature of calpain aggregation.

MATERIALS AND METHODS

Protein Expression and Purification—Both m-calpain and \(\mu\)-calpain consist of an 80-kDa large subunit, which has an ~62% sequence identity between the isoforms, and a 28-kDa small subunit, which is identical in the two isoforms. Upon Ca\(^{2+}\)-induced activation, calpain undergoes autolysis in both subunits. The natural rat calpain small subunit contains 270 residues, of which the N-terminal glycine-rich region of ~83 residues (domain V) is unstructured in the crystal structure (13) and is rapidly removed by autolysis (20). In this work, our recombinant calpains contain either a 21-kDa small subunit of 184 residues corresponding closely to the natural small subunit autolysis product (20, 21), or an N-terminal His-tag version of this 21-kDa subunit containing 205 residues. The absence of domain V and residues (domain VI), corresponding closely to the natural small subunit together with an amorphous aggregate enriched in the large subunit. The mother liquor showed faint traces of the 21-kDa subunit. The volume of 3.0 ml used for conventional light-scattering required too much material for studies at a higher protein concentration. Therefore, dynamic light-scattering experiments were performed at 20 °C using the DynaPro-MSX instrument (Protein Solutions. Charlottesville, VA), which has an operating volume of 1 ml.

Analytical Ultracentrifugation—The volume of 3.0 ml used for conventional light-scattering required too much material for studies at a higher protein concentration. Therefore, dynamic light-scattering experiments were performed at 20 °C using the DynaPro-MSX instrument (Protein Solutions. Charlottesville, VA), which has an operating volume of 1 ml.

RESULTS

Crystalization—Following the preincubation of C105S-m-80kCHis6/21k in the presence of 10 mM Cl\(_2\) in a Beckman XL-1 analytical ultracentrifuge using absorbance optics following the procedure described by Laue and Stafford (23). Protein solutions were exhaustively dialyzed against 50 mM Tris-HCl, pH 7.6, containing 200 mM NaCl, 2 mM tri-(2-carboxyethyl)-phosphine, and either 5 mM EDTA or 5 mM CaCl\(_2\). Runs were performed at a protein concentration of 1.13 mg/ml both in the presence and absence of Ca\(^{2+}\) at either 50,000 or 60,000 rpm for 3.5 h, during which time a minimum of 50 scans was taken to monitor the sedimentation rate of the protein. For sedimentation equilibrium, experiments were performed at three different protein concentrations of 0.09—0.3 mg/ml and at a minimum of two different speeds between 6000—12,000 rpm. Each speed was maintained until there was no significant difference from \(t = 72\) min and the time-dependent change in scattering intensity was recorded. The solutions contained 70—200 \(\mu\)g/ml of calpain, 0—0.2% NaCl, 10 mM dithiothreitol, and 20—330 mM buffer, in a total volume of 3.0 ml. The buffer system was varied to alter the pH, and the CaCl\(_2\) concentration was varied from 0.1—50 mM.

Aggregation and Calpastatin-Calpain Complex Formation—At low ionic strength, calpain aggregated on the addition of sufficient Ca\(^{2+}\) and this could be reversed rapidly and completely by excess EDTA and more slowly reversed by raising the NaCl concentration (Figs. 2, a and b). In buffers containing 70\(\mu\)g/ml calpain and 0.2 mM NaCl, no detectable increase in light scattering was observed. The solutions contained 70–200 \(\mu\)g/ml of calpain, 0–0.2 mM NaCl, 10–200 mM dithiothreitol, and 20–330 mM buffer, in a total volume of 3.0 ml. The buffer system was varied to alter the pH, and the CaCl\(_2\) concentration was varied from 0.1—50 mM.
scattering was caused by the addition of Ca\(^{2+}\). Aggregation at lower ionic strength was also prevented by the presence of calpastatin, and in this case, the small increase in light scattering was assumed to mark the onset of the calpastatin-calpain complex formation (Fig. 2c). The calpastatin used here was a recombinant form of rat calpastatin domain I containing a total of 140 residues (22). Table I lists the Ca\(^{2+}\) requirements for these two different events and shows that aggregation of C105S-m-80k/21k calpain was dependent on the ionic strength and pH of the buffer as well as on the Ca\(^{2+}\) concentration. The Ca\(^{2+}\) concentration required in any given buffer for calpastatin-calpain complex formation was significantly higher than that required for aggregation (24).

Dynamic light-scattering experiments were conducted at higher calpain concentrations than the light-scattering work in order to approach conditions relevant to crystallization trials. They showed that a solution of 1 mg/ml of m-calpain in 50 mM Tris-HCl, pH 7.6, was monodisperse in the absence of Ca\(^{2+}\). The major species had a molecular mass of 95–105 kDa, and ~5% of the protein was present as high molecular mass species in the range of at least 10^5 kDa. At this low ionic strength, the solution became polydisperse upon the addition of Ca\(^{2+}\) but remained monodisperse in 0.5 M NaCl with Ca\(^{2+}\). With 3–5 mg/ml calpain in 1 M NaCl in the presence of Ca\(^{2+}\), 50–70% of the scattering intensities were contributed by the monomer of ~100 kDa, and the remainder were provided by components of the order of 10^3–10^5 kDa. The data did not indicate the presence of the homodimer of 21 kDa.

**Ultracentrifugation Studies**—Sedimentation studies also provided clear evidence of aggregation. Protein samples (1.13 mg/ml) gave rise to a sedimentation coefficient value (s_{20,w}) of 5.93 in the absence of Ca\(^{2+}\), whereas in the presence of Ca\(^{2+}\), the coefficient value was 17.79. In the sedimentation equilibrium experiment, m-calpain (0.09–0.3 mg/ml) in the absence of Ca\(^{2+}\) showed a single molecular species of 100 kDa, but in the presence of Ca\(^{2+}\), multiple species were observed with molecular masses ranging from 276 to 600 kDa, suggesting the presence of a mixture of aggregates of 4–8 calpain molecules or calpain large subunits. The data again did not provide evidence for the existence of the isolated small subunit. As a control, the isolated 21-kDa small subunit protein, which is known to exist as a homodimer (25), was analyzed at the same time under the same conditions. In the absence of Ca\(^{2+}\), it was monodisperse with an apparent molecular mass of 40 kDa. In the presence of Ca\(^{2+}\), it was polydisperse with a dominant species of an apparent molecular mass of 47.5 kDa.

**Subunit Dissociation**—The subunit exchange experiment depended on two factors, the separation by ion exchange chromatography of the \(\mu\)-calpain-like m-Bam-C115S-\(\mu\)-Dra-m-80k/
The calpain concentration was constant at 70 μg/ml (0.7 μM), and calpastatin (a recombinant form of rat calpastatin domain I of 148 residues) was added at a concentration of 1.4 μM. The Ca\(^{2+}\) requirement was estimated from the point at which an abrupt change in light scattering occurred during stepwise addition of Ca\(^{2+}\). The experiments were carried out at 20 °C.

### Table I

| Buffer conditions         | Ca\(^{2+}\) requirement for calpain aggregation | Ca\(^{2+}\) requirement for calpastatin-calpain complex formation |
|---------------------------|-----------------------------------------------|-----------------------------------------------------------------|
| 0.1 M MES, pH 6.25        | 0.2                                           | 0.3–0.5                                                         |
| 0.1 M MES, pH 6.25, 0.1 M NaCl | no                          | no                                                             |
| 0.33 M MES, pH 6.25      | no                                           | no                                                             |
| 0.1 M HEPES, pH 7.0       | no                                           | no                                                             |
| 20 mM HEPES, pH 7.5       | 0.8                                          | 2–3                                                            |
| 0.1 M HEPES, pH 7.5       | 3                                            | 5                                                              |
| 20 mM Tris-HCl, pH 7.6    | 1–2                                          | 5–6                                                            |
| 100 mM Tris-HCl, pH 7.6   | no                                           | no                                                             |

**FIG. 3.** Fast Protein Liquid Chromatography separation of calpain isoforms on a 1 ml UnoQ (Bio-Rad) quaternary ion exchange column. The earlier peak contained m-Bam-C1155-S-μ-Dral m-80k/NHis\(_{10-21}\)k calpain, and the later peak contained C105S-m-80k/21k calpain. The column was run in 50 mM Tris-HCl, pH 7.6, 2 mM EDTA, 0.1% (v/v) Triton X-100, 10 mM 2-mercaptoethanol (buffer A) with a gradient of increasing NaCl concentration. a, a mixture of the two proteins resolved without previous exposure to Ca\(^{2+}\). b, a mixture of the two proteins resolved following exposure to 5 mM Ca\(^{2+}\) for 30 min, quenching with excess EDTA, and dialysis overnight against buffer A.

**FIG. 4.** Immunoblot analysis of the eluted peaks shown in Fig. 3. Fractions spanning the two main eluted peaks in each column were analyzed by means of immunoblotting. The left-hand section of the blot contains samples from the column (see Fig. 3a) previous to Ca\(^{2+}\) treatment, and the right-hand section contains samples from the column (see Fig. 3b) following exposure to Ca\(^{2+}\). The upper portion of the blot was treated with a mixture of a polyclonal antibody to rat m-calpain large subunit and a monoclonal antibody to human μ-calpain large subunit, which cross-reacts with rat μ-calpain. The lower portion of the blot was treated with a polyclonal antibody to the rat calpain small subunit. Both blots were treated with appropriate second antibodies and then developed by enhanced chemiluminescence. It is important to note the appearance of the NHis\(_{10-21}\)k subunit in the m-calpain peak and the corresponding appearance of the 2kDa subunit in the μ-calpain peak only after Ca\(^{2+}\) treatment as indicated by open arrows.

**DISCUSSION**

The impetus for this work was the need to crystallize calpain in the presence of Ca\(^{2+}\). The structure of calpain in the absence of Ca\(^{2+}\) (12, 13) showed that the active site was not assembled to a catalytically active conformation, so that it was clearly of interest to solve the structure in the presence of Ca\(^{2+}\) to understand the mechanism of calpain activation. Not unexpectedly, however, crystallization in the presence of Ca\(^{2+}\) raised problems of aggregation and subunit dissociation. The suggestion that the calpain subunits dissociate in the presence of Ca\(^{2+}\) has been the subject of some debate (15–19) arising not least from the difficulty of designing definitive experiments. Here we provide evidence that clearly confirms the phenomenon of subunit dissociation first described by Yoshizawa et al. (15) in 1995. The results also suggest that in m-calpain and in the absence of autolysis, the aggregation/dissociation is a highly reversible process. The dissociation is normally not complete unless driven by other factors. Extrapolation of these clear in vitro results to the cell is necessarily speculative. It seems highly probable that the subunit dissociation is an essential aspect of calpain activation in vivo, but the ensuing aggregation observed in vitro is less likely to be relevant in vivo where calpain is diluted in a highly proteinaceous environment and where autoylation rapidly removes the activated calpain.

Calpain aggregation is clearly a function of protein concentration, ionic strength, and Ca\(^{2+}\) concentration, but some new insights into the aggregation were obtained from light-scattering and ultracentrifugation experiments. First, within the experimental time frame (~1 h) and in the absence of autolysis, aggregation is an equilibrium process, because it could be fully reversed by the addition of EDTA. Second, the inhibition of aggregation by higher salt concentration strongly suggests that...
hydrophobic interactions are involved. Although aggregation of calpain at <200 μg/ml appeared to be suppressed by 0.2 M NaCl, both dynamic light scattering and ultracentrifugation run at ≥ 1-mg/ml calpain showed that soluble high molecular weight oligomers were formed in the presence of Ca\(^{2+}\), which could only partly be suppressed by 0.5 or 1 M NaCl. Both of these methods failed to detect the presence of the isolated 21-kDa homodimer, which was anticipated as a result of subunit dissociation. Previous work on the 21-kDa subunit showed that it exists as a homodimer both in the presence and absence of Ca\(^{2+}\), and that the homodimer once formed is unlikely to dissociate in any of the conditions used here (25). The absence of free small subunits in the light-scattering and centrifugation experiments therefore suggests that subunit dissociation is far from complete in these conditions, and that the large soluble complexes are still composed predominantly of calpain (80 + 21 kDa) heterodimers, which have undergone Ca\(^{2+}\)-induced conformational changes leading to aggregation. It is possible to imagine a highly flexible intermediate form of the heterodimer in which the contact between the N-terminal peptide of the large subunit and domain VI in the small subunit has been lost, whereas the subunits remain at least partially attached through residual contacts between domains IV and VI.

A third aspect of the aggregation studies was the inhibition of aggregation by a molar excess of a 140-residue domain of calpastatin. Separate sections of calpastatin are known to bind to domain IV in the large subunit and to domain VI of the small subunit (26). Therefore, it seems probable that calpastatin prevents calpain aggregation by binding to both subunits simultaneously and preventing subunit dissociation.

 Whereas m-calpain dissociation appears to be incomplete in many buffer conditions, it is clear that calpain does indeed dissociate in the presence of Ca\(^{2+}\) at pH 6.5 in the conditions prevailing in some crystallization droplets. In these precipitant conditions, the dissociated 21-kDa small subunit crystallized out, and its removal presumably promoted further subunit dissociation, leaving the large subunit to precipitate as an amorphous aggregate. Several crystallization conditions have also been found in which no visible aggregation of calpain occurs in the presence of Ca\(^{2+}\), but useful crystals, whether they are of the activated heterodimer or of the isolated large subunit, have not yet been obtained. Finally, the subunit exchange experiment also clearly demonstrated reversible subunit dissociation of calpain, because the interchange of small subunits between the m- and μ-type is only possible provided they dissociate. We had earlier failed to detect subunit dissociation by means of column chromatography of calpain in the presence of Ca\(^{2+}\) and by a less definitive version of the subunit exchange experiment (19), but the present experiments provide unequivocal proof of subunit dissociation.

The importance of hydrophobic interactions and the effects of calpastatin support the idea that aggregation is a result of subunit dissociation. The crystal structure of calpain shows that the large and small subunits in the absence of Ca\(^{2+}\) are bound mainly by interactions between the fifth EF-hand motif of each subunit (12, 13) in a manner very similar to that shown for the 21-kDa homodimer (25, 27). Dissociation must expose the complementary dimerization interface on both subunits, which is a large hydrophobic area of ~2780 Å\(^2\) representing approximately one quarter of the surface area of domain IV and equally of domain VI (Fig. 5). Such an exposure is energetically unfavorable in aqueous solution and would be expected to promote either “correct” reassociation of the large and small subunits or random association leading to aggregation. The exposure of this surface would be even more disfavored in solutions of higher ionic strength, which explains the partial suppression of calpain aggregation in 0.2–1 M NaCl. Our modeling suggests that two large subunits could not dimerize at this site in domain IV in their correct relative orientation as observed in the domain VI homodimer because of steric interference by domains I–III. Thus, even partial exposure of this hydrophobic patch would promote the formation of randomly associated aggregates.

Based on the first x-ray structure of m-calpain (12), we proposed that the constraints imposed upon the protease domains I and II by the remainder of the molecule would act as a barrier in the activation of calpain. These constraints were provided on the one side by the small subunit domain VI, binding to the large subunit N-terminal peptide of domain I, and on the other side by a set of salt links between domains II and III. The release of the constraints would facilitate the assembly of the competent catalytic triad. We have reported experiments supporting the idea of the domain II/domain III interaction (14), and this work suggests that dissociation of the small subunit would release the constraint on domain I.

The evidence of aggregation, calpastatin complex formation, and subunit dissociation suggests that subunit dissociation is
the principal cause of Ca$^{2+}$-induced aggregation of calpain. Furthermore, we also show that both dissociation and aggregation are reversible. Our present understanding of these events is shown in Fig. 5. Ca$^{2+}$-induced conformational rearrangement and partial dissociation leads to the formation of aggregates probably containing heterodimers as well as dissociated large subunits. This step is largely reversible, but the small subunit homodimer is probably no longer available for reassociation, and larger aggregates composed almost exclusively of large subunit will be formed almost irreversibly. In the case of active calpains, aggregation also occurs in vitro on initial exposure to Ca$^{2+}$, but autolysis at the same time begins to degrade the large subunits to inactive fragments.

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