Calpain Mutants with Increased Ca\textsuperscript{2+} Sensitivity and Implications for the Role of the C\textsubscript{2}-like Domain\textsuperscript{*}

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The ubiquitous calpain isoforms (\(\mu\)- and m-calpain) are Ca\textsuperscript{2+}-dependent cysteine proteases that require surprisingly high Ca\textsuperscript{2+} concentrations for activation in vitro (\(-50\) and \(\sim 300 \mu\text{M}\), respectively). The molecular basis of such a high requirement for Ca\textsuperscript{2+} in vitro is not known. In this study, we substantially reduced the concentration of Ca\textsuperscript{2+} required for the activation of m-calpain in vitro through the specific disruption of interdomain interactions by structure-guided site-directed mutagenesis. Several interdomain electrostatic interactions involving lysine residues in domain II and acidic residues in the C\textsubscript{2}-like domain III were disrupted, and the effects of these mutations on activity and Ca\textsuperscript{2+} sensitivity were analyzed. The mutation to serine of Glu-504, a residue that is conserved in both \(\mu\)- and m-calpain and interacts most notably with Lys-234, reduced the in vitro Ca\textsuperscript{2+} requirement for activity by almost 50%. The mutation of Lys-234 to serine or glutamic acid resulted in a similar reduction. These are the first reported cases in which point mutations have been able to reduce the Ca\textsuperscript{2+} requirement of calpain. The structures of the mutants in the absence of Ca\textsuperscript{2+} were shown by x-ray crystallography to be unchanged from the wild type, demonstrating that the increase in Ca\textsuperscript{2+} sensitivity was not attributable to conformational change prior to activation. The conservation of sequence between \(\mu\)-calpain, m-calpain, and calpain 3 in this region suggests that the results can be extended to all of these isoforms. Whereas the primary Ca\textsuperscript{2+} binding is assumed to occur at EF-hands in domains IV and VI, these results show that domain II–domain III salt bridges are important in the process of the Ca\textsuperscript{2+}-induced activation of calpain and that they influence the overall Ca\textsuperscript{2+} requirement of the enzyme.

The two ubiquitous calpains, \(\mu\)- and m-calpain, are cytosolic thiol proteases entirely dependent on Ca\textsuperscript{2+} for their activity. These two calpains consist of a large or catalytic subunit (80 kDa) (from the genes \textit{capn1} and \textit{capn2}, respectively) and a common small or regulatory subunit (28 kDa) (from \textit{capn4}). The \(\mu\)- and m-isofoms differ also in the concentration of Ca\textsuperscript{2+} required for half-maximal activation in vitro. Both enzymes require a Ca\textsuperscript{2+} concentration (\(-50\) and \(\sim 300 \mu\text{M}\) for \(\mu\)- and m-calpain, respectively) that is significantly higher than that available in vivo (<1 \(\mu\text{M}\)). Autolysis causes a drop in Ca\textsuperscript{2+} requirement, but calpain activation in vitro must involve additional factors, such as membrane-binding and activator proteins. Although the physiological roles of these calpains remain unclear, there is much evidence suggesting that they contribute to many cellular processes, including signal transduction, apoptosis, cell cycle regulation, and cytoskeletal reorganization (1–5). Their physiological importance is exemplified by the recent demonstration that transgenic mice lacking the classical calpain isoforms die during embryonic development (6). Excessive proteolysis by these enzymes in response to altered Ca\textsuperscript{2+} homeostasis has been observed in several neuropathological states, including Alzheimer’s disease (7, 8). With regard to other forms of calpain, defects in calpain-3 lead to the development of limb-girdle muscular dystrophy 2A (9), calpain-10 is linked to type II diabetes (10), and in \textit{Caenorhabditis elegans}, the protease activity of the calpain homologue TRA-3 is required for sex determination (11, 12).

The x-ray structures of rat (13) and human (14) m-calpain in the absence of Ca\textsuperscript{2+} show that the large subunit consists of a short a-helix at the N terminus; domains I and II, which constitute the protease function; domain III, which resembles a C\textsubscript{2}-domain; and domain IV, which contains several EF-hands. The small subunit contains domain V, which is glycine- and proline-rich and was either not present (13) or not detectable (14) in the x-ray structure, and domain VI, which contains several EF-hands and is structurally very similar to domain IV. Importantly, the structure revealed that the protease active site is not assembled in the absence of Ca\textsuperscript{2+}, a feature not previously observed in any other cysteine protease. The protease domains (domains I and II) are held apart in the Ca\textsuperscript{2+}-free conformation by interactions within the molecule, which prevent the catalytic triad residues (Cys-105 in domain I and His-262 and Asn-286 in domain II) from assuming the correct conformation to hydrolyze substrates. Activation by Ca\textsuperscript{2+} must somehow relieve these constraints, permitting domains I and II to move toward each other and form a competent active site. A Ca\textsuperscript{2+}-bound structure of calpain might answer several questions concerning the Ca\textsuperscript{2+}-induced activation mechanism, but this structure has not yet been determined because of significant technical difficulties.

Based on the primary sequence alone, it was for a long time assumed that the EF-hand-containing domains IV and VI were the major determinants of the Ca\textsuperscript{2+} requirement of calpain. Recent studies have shown that Ca\textsuperscript{2+} binding at EF-hand 3 makes the largest contribution to calpain activation, EF-hand 2 may make some contribution, and the other EF-hands apparently have no direct role in Ca\textsuperscript{2+} regulation (15). Additionally, it has recently been demonstrated that TRA-3 has Ca\textsuperscript{2+}-dependent protease activity although it lacks the EF-hand domain (12). Thus it is likely that the observed Ca\textsuperscript{2+} requirement

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phospholipid binding of C2-containing proteins (16, 17). This is domain III. Dotted lines and several other acidic residues (as well as Lys-354, form several interdomain salt bridges with Glu-504 by Ca2+ are thought to translocate to the cell membrane when activated consistent with the following two considerations: 1) calpains reduced in the presence of phospholipids, which appears to be domain II (Fig. 1), composed of Glu-392–Asp-400 and Glu-504 in domain III and fore that this salt bridge region exerts a conformational con-

First, domain III was found to be structurally similar to the C2-like domain III and protease domain II, A, overall view of the m-calpain structure in the absence of Ca2+. The domain classification is taken from Ref. 13. The region contained within the black circle is amplified for clarity in B. B, electrostatic interactions between domains II and III. Lysine residues (dark blue) Lys-226, Lys-230, and Lys-234 on a domain II α-helix (cyan), as well as Lys-354, form several interdomain salt bridges with Glu-504 and several other acidic residues (red) on one loop (residues 392–400) of domain III. Dotted lines, possible interactions with Glu-504. In this study, Lys-226, Lys-230, Lys-234, and Glu-504 were mutated to determine the effects that disruption of these interdomain interactions might have on the activation of m-calpain by Ca2+. This figure was prepared using MOLSCRIPT (23) and RASTER3D (24).

of the whole enzyme is not determined solely by the EF-hands and is greatly affected by interactions elsewhere in the molecule.

The x-ray structures revealed two interesting features in domain III that may be very important in this regard (Fig. 1A). First, domain III was found to be structurally similar to the Ca2+-dependent C2 domains, which are known to promote the phospholipid binding of C2-containing proteins (16, 17). This is consistent with the following two considerations: 1) calpains are thought to translocate to the cell membrane when activated by Ca2+ and 2) the Ca2+ requirement of calpain is greatly reduced in the presence of phospholipids, which appears to be a part of the in vivo activation mechanism of calpain. Second, at the interface between domains II and III, there is a set of electrostatic interactions involving a remarkably acidic loop composed of Glu-392–Asp-400 and Glu-504 in domain III and clustered lysine residues Lys-226, Lys-230, and Lys-234 in domain II (Fig. 1B). All these residues are highly conserved within the known m-calpain sequences. We hypothesize therefore that this salt bridge region exerts a conformational constraint on the movement of domain II and therefore on the assembly of the active site, which will tend to elevate the Ca2+ requirement of the enzyme. By disrupting these salt bridges, the mutations described here were designed to investigate the effects on the Ca2+ requirement of calpain. Our experiments would further address the question of whether these electrostatic interactions directly contribute to maintaining the inactive conformation of calpain.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis and Protein Expression—Site-directed mutagenesis was performed on single-stranded DNA derived from pET-24-m-80k-CHis6, using the following antisense primers: 5’-P-cttgcttgtagc-gagacgacgagc-gagacc (K226S), 5’-P-agagacctctctctctctctctctctctctctctctctctc (K230S), 5’-P-gaagagacaagcagagagagatgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtg
matographic columns to ~90–99% purity based on Coomassie staining of SDS-polyacrylamide electrophoresis gel, depending on the mutant (Fig. 2).

Effect of Mutations on Ca$^{2+}$ Requirement and Specific Activity—Enzymes were assayed for their ability to hydrolyze casein at varying concentrations of Ca$^{2+}$. The calculated [Ca$^{2+}$]$_{0.5}$ served as the basis for the comparison of the Ca$^{2+}$ requirement of wild-type and mutant enzymes in this study. In the conditions employed here, the [Ca$^{2+}$]$_{0.5}$ for wild-type m-calpain was 242 ± 6 μM (Fig. 3), which is in general agreement with previous reports (1–5). Introducing the mutation E504S in domain III had the most dramatic effect, reducing the [Ca$^{2+}$]$_{0.5}$ to 129 ± 1 μM, corresponding to a 47% reduction in the Ca$^{2+}$ requirement compared with wild-type m-calpain. The effects of mutating each of the three lysine residues (Lys-226, Lys-230, and Lys-234) individually to serine had varying results. Mutating Lys-226 or Lys-230 had minor effects on the Ca$^{2+}$ sensitivity of the enzyme (Fig. 3, Table I), whereas the mutation of Lys-234 reduced the [Ca$^{2+}$]$_{0.5}$ to 183 ± 1 μM (a 24% reduction in the Ca$^{2+}$ requirement). Further, the mutation of Lys-234 to glutamic acid resulted in a reduction in the [Ca$^{2+}$]$_{0.5}$ to 159 ± 3 μM, corresponding to a 34% decrease. Interestingly, the K230E and K234E mutations decreased the specific activity of the enzyme to ~16% compared with the wild type, whereas the K230S and K234S mutations had no significant effect on specific activity.

X-ray Crystallography—To ascertain whether mutations had caused unexpected structural changes that might influence the Ca$^{2+}$ sensitivity, we attempted structure determination of these mutants by x-ray crystallography. We were successful in crystallizing K226S, K230E, K234S, and E504S mutants in the absence of Ca$^{2+}$ in conditions very similar to those for wild-type rat m-calpain (19). To date, we have solved the structures of the K230E and E504S mutants, from which larger crystals suitable for diffraction were obtained. The refined structures of these mutants are virtually indistinguishable from wild-type calpain (root mean square deviation of 0.47 and 0.49 Å on α-carbon atoms for the E504S mutant and the K230E mutant, respectively), even in the vicinity of the mutations. Because these two structures were almost identical to the wild type, we did not proceed with the mutants that only gave rise to weakly diffracting crystals and assumed that they would have minimal, if any, conformational changes as well.

**DISCUSSION**

The mechanism of calpain activation by Ca$^{2+}$ is a fundamental biochemical question that has remained poorly understood. A particularly interesting feature of these enzymes is that their **in vitro** requirements for Ca$^{2+}$ are orders of magnitude higher than typical Ca$^{2+}$ concentrations found in the cell. This characteristic is not shared by similar members of the EF-hand family, such as calmodulin, which have **in vitro** Ca$^{2+}$ requirements within the physiological range. Attempts to convert calpain EF-hand sequences to more canonical forms have been unsuccessful in reducing the Ca$^{2+}$ requirement (15), suggesting that other structural features contribute to calpain’s requirement for Ca$^{2+}$. The x-ray structure of Ca$^{2+}$-free rat m-calpain revealed several features that appear to maintain the inactive conformation of the enzyme. Of particular interest is a loop of nine acidic residues (Glu-392–Asp-400) in domain III, together with Glu-504, which make several electrostatic contacts with three lysine residues (Lys-226, Lys-230, Lys-234) on one α-helix in domain II. The structure of human m-calpain showed an additional interaction between Glu-504 and Lys-354. It should be noted particularly that Glu-504 would have the strongest additional interaction between Glu-504 and Lys-354. It should therefore seemed attractive to propose that this set of salt bridges might affect the ability of domain II to approach domain I in the process of assembling the active site, leading to the activation of calpain.

The K234S and E504S mutations both significantly reduced the Ca$^{2+}$ requirement without affecting the specific activity of the enzyme. The reduction caused by the E504S mutation was
greater than that caused by the K234S mutation. This suggests that Glu-504 makes multiple electrostatic contacts and probably reflects the fact that the E504S mutation abolishes the salt links to Lys-234 and Lys-354. The K234S mutation abolishes only one of these salt links. The crystal structure of E504S is virtually identical to that of the wild-type enzyme in the inactive state, demonstrating that the reduction in the Ca\(^{2+}\) requirement observed in E504S is not a result of the disruption of the inactive conformation but must involve the facilitation of domain movement during Ca\(^{2+}\) activation. Another interesting result was observed with the K234E mutation, which reduced the Ca\(^{2+}\) requirement of the enzyme even further than the K234S mutation. This mutation also reduced the specific activity of the enzyme, illustrating the sensitivity of this region to changes in the electrostatic potential. This mutant was expected to exert a repulsive interaction with Glu-504.

Surprisingly, the results of this work showed that the electrostatic interactions of Lys-226 and Lys-230 with the acidic Glu-392–Asp-400 loop do not appear to be critical to the activation of calpain by Ca\(^{2+}\) because the K226S and K230S mutations did not affect either the Ca\(^{2+}\) requirement of the enzyme or its specific activity. The K230E mutation, which is expected to introduce a strong repulsion between this position and the acidic loop, did not affect the Ca\(^{2+}\) requirement of the enzyme. It did, however, greatly reduce the specific activity of the enzyme, for reasons that we cannot presently explain. The crystal structure of K230E in the absence of Ca\(^{2+}\) was identical to that of wild-type m-calpain, showing that the mutation did not affect the domain II-domain III geometry in the resting state of the enzyme. Because it has not so far been possible to crystallize calpain in the presence of Ca\(^{2+}\), the effects of these mutations on the activated state of the enzyme cannot be directly observed.

Of the many mutations that have been made in m-calpain, the Lys-234 and Glu-504 mutations described here are the only examples so far described that result in a lowering of the Ca\(^{2+}\) requirement. The question therefore arises of how a loss of salt linkages around Glu-504 can have a major effect whereas alteration at Lys-226 and Lys-230 has very little effect. The residue Glu-504 is at one end of the “transducer arm” or linker peptide that connects domain IV to domain III, and this arm is assumed to communicate Ca\(^{2+}\)-induced conformation changes in domain IV to the rest of the molecule. The evidence therefore suggests that the loss of the salt bridges at Glu-504 makes the movement of domain II toward domain I “easier,” i.e., permits it to occur at a lower Ca\(^{2+}\) concentration. As can be seen in Fig. 1, Glu-504 is “strategically” located at the top right end of the salt bridge series. The movement of domain II in the assembly of the active site, according to our modeling studies guided by the many well established thiol protease structures, would be based on a pivotal point in the Glu-504 region. Therefore, given that the Glu-504 and Lys-234 mutations weaken the interaction of domain II with domain III, we would suggest that domain II does in fact move apart from domain III during the activation process. Whether domain III undergoes additional conformational changes remains unknown in the absence of a Ca\(^{2+}\)-bound structure. Illustrating the importance of this interaction in the calpain family, the corresponding mutation in m-calpain, E515S, also lowers the Ca\(^{2+}\) requirement (data not shown).

Recalling the resemblance of domain III to C\(_2\) domains, it should also be noted that Glu-504 and the Glu-392–Asp-400 loop are in positions very similar to the loops in C\(_2\) domains that participate in Ca\(^{2+}\)-dependent phospholipid binding. The Ca\(^{2+}\) requirement of calpain is greatly reduced in the presence of some phospholipids in vitro, and this is assumed to reflect membrane binding in vivo. We speculate therefore that Ca\(^{2+}\) and phospholipid binding to this region of calpain could disrupt these critical salt links, thereby releasing some constraints on the movement of domain II and lowering the Ca\(^{2+}\) requirement of the enzyme. Coordination of Ca\(^{2+}\) in the acidic loop by the side chains of the acidic residues could break most or even all of the electrostatic interactions with the basic residues in domain II. With the exception of pivotal Glu-504, perhaps breaking one or two such salt links by point mutation may not be enough to completely relieve the conformational constraints to permit the movement of domain II.

Given that a Ca\(^{2+}\)-bound structure may not be available in the foreseeable future, experiments of the type employed here will be required to more fully understand the mechanism of calpain activation by Ca\(^{2+}\). In this work, we have for the first time generated calpain mutants that are responsive to significantly lower concentrations of Ca\(^{2+}\) for activation. Further, we have identified a specific structural feature remote from the EF-hand domains that affects the Ca\(^{2+}\) sensitivity and activation of calpins. In light of the sequence conservation displayed in this region, it is clear that these key interdomain interactions are important factors contributing to the overall Ca\(^{2+}\) requirement of the calpins.

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