Multiple interactions of the 'transducer' govern its function in calpain activation by Ca^{2+}

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Typical calpains in mammals become activated on binding of 8–12 Ca^{2+} ions per enzyme molecule, giving an example of integrated, manifold regulation by calcium. Besides two identified Ca^{2+} sites in catalytic domain II and several EF-hand motifs in domains IV and VI, an acidic loop in the centrally positioned domain III seems to harbour Ca^{2+}. The mediator of distant Ca^{2+}-induced structural transitions is an elongated structural element, the 'transducer'. By site-directed mutagenesis along the transducer, we have generated various forms of rat m-calpain in which critical intramolecular interactions, as judged from the X-ray structure, would be abolished or modified. The kinetic parameters of these mutant enzymes support a model featuring shrinkage of transducer as a contributor to structural changes involved in calpain activation.

Key words: calcium, calpain, extended transducer, intramolecular signal propagation, kinetic analysis, site-directed mutagenesis.

INTRODUCTION

The ubiquitous (typical) mammalian calpains (μ- and m-calpains), just as their *Drosophila* homologues, Calpains A and B are calcium-activated cytoplasmic thiol-proteases [1–4]. Depending on the enzyme form and origin, they bind 8–12 Ca^{2+} ions, which induces a structural rearrangement in the enzyme resulting in the shaping of the active site, i.e. proper juxtaposition of cysteine, histidine and asparagine residue side chains. Distinct elements of this transition have been uncovered by recent efforts. A major obstacle to finalizing the picture in all details has been the lack of a high-resolution X-ray structure of the Ca^{2+}-bound, active form of calpain. On the basis of indirect evidence, the high-resolution structure of the Ca^{2+}-free, inactive enzyme [5,6] and the solution of partial Ca^{2+}-bound structures, the following picture has emerged.

Ca^{2+}-binding to the calmodulin-like domains of both the large and small subunits triggers a cascade of structural changes that culminate in the formation of the catalytically competent conformation. The binding of Ca^{2+} to these domains (IV and VI) elicits small [7,8] but significant [9] conformational changes in this region. These changes probably travel along two paths towards the active site, on opposite sides of the molecule. Domain IIa is positioned in the inactive enzyme by a helical anchor (domain I [6] or anchor peptide [5]) that sits in a groove on the surface of domain VI. This anchor is released on activation facilitated by autolytic cleavage and liberates domain IIa [10] (but see also [11]). Domain IIb is mostly positioned by multiple salt bridges between its basic surface cluster and an acidic loop in domain III, composed of 8–10 aspartic or glutamic residues. These salt bridges are loosened by the pulling of an extended transducer that connects domains III and IV [12] and Ca^{2+} binding at the acidic loop itself [13,14]. The removal of the structural constraints on both sides of the catalytic domain enables the closure of the active site.

The breakthrough with respect to the mechanism of this closure came when the 'core-enzyme' consisting solely of the catalytic domain II lent itself to X-ray analysis in the presence of Ca^{2+} [15,16]. These experiments revealed that, in this 'minicalpain', the active-site cleft is closed so that the catalytic residues (cysteine, histidine and asparagine) are in appropriate proximity to make a papain-like active site. Another dividend was the discovery of two non-EF-hand Ca^{2+}-binding sites within domain II, which had to be occupied by Ca^{2+} for activity. Although the specific activity of the 'core-enzyme' was low, these sites certainly play a crucial role in calpain activation.

Besides X-ray diffraction, site-directed mutagenesis at critical points of the enzyme molecule and the kinetic analysis of mutant enzymes, provides valuable information on the fine details of the activating structural transition. This approach, although yielding only indirect structural information, has the virtue of reporting on the functioning of the enzyme in solution, without any constraints imposed by the crystal lattice. In a previous work [12], we analysed the functional responses of rat m-calpain and *Drosophila* Calpain B to exchange (deletion) of selected amino acids in the sequence. We paid particular attention to the acidic loop in domain III, and concluded that a long peptide segment named the transducer [5] forms a structural unit with the acidic loop, which may contribute to the fine-tuning of the structural transition ('extended transducer' [12]). In the present study, we scrutinized the role of the transducer in more detail, by replacing several of the residues involved in intramolecular salt bridges. The emerging picture is in accordance with, or complementary to, data obtained in our and other laboratories.

EXPERIMENTAL

Materials and general methods

The expression vectors encoding rat m-calpain large and small subunits (pET-24-m-80k-$CHis_6$ and pACpET-21k respectively) were provided by Professor J. S. Elce (Department of Biochemistry, Queen's University, Kingston, ON, Canada).

The restriction enzymes and protein marker were obtained from New England Biolabs (Beverly, MA, U.S.A.). The plasmid preparation kit (NucleoSpin®) was from Macherey-Nagel (Düren, Germany). For the development of mutants, QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) was used. DNA sequencing was performed by MWG Biotech AG (Ebersberg, Germany). For protein expression, *Escherichia coli* BL21(DE3) strain from Novagen (Madison, WI, U.S.A.) was

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Table 1 Oligonucleotides used for site-directed mutagenesis of rat m-calpain

Mutation	Sequences of the forward primers
K505S	5′-CCGAGTCTTCTCAGAGAGCAAGGCTGACTACCAAAC-3′
K505A/K506S	5′-CCGAGTCTTCTCAGAGGCCAGCGCTGACTACCAAACTGTCG-3′
K505S/K506S	5′-GCATCCGAGTCTTCTCAGAGAGCAGCGCTGACTACCAAACTGTCGATG-3′
D513N/D514N	5′-GGCTGACTACCAAACTGTCAATAATGAAATCGAGGCCAACATTG-3′
D513G/D514G/E515G	5′-GCTGACTACCAAACTGTCGGTGGTGGCATCGAGGCCAACATTG-3′
E521Q/E522Q	5′-GAAATCGAGGCCAACATTCAACAGATTGAAGCCAATGAGGAGG-3′

Table 2 Partial amino acid sequences around mutated sites in m-calpain

New residues are shown in bold; (i)–(iv) indicate mutations in different regions.

	Mutants	Sequences
(i)	Wild-type	IRVFSEKKADYQTVDDEIEANIEEIEANEE
(ii)	K505S	IRVFSE**S**KADYQTVDDEIEANIEEIEANEE
	K505A/K506S	IRVFSE**AS**ADYQTVDDEIEANIEEIEANEE
	K505S/K506S	IRVFSE**SS**ADYQTVDDEIEANIEEIEANEE
(iii)	D513N/D514N	IRVFSEKKADYQTV**NN**EIEANIEEIEANEE
	D513G/D514G/E515G	IRVFSEKKADYQTV**GGG**IEANIEEIEANEE
(iv)	E521Q/E522Q	IRVFSEKKADYQTVDDEIEANI**QQ**IEANEE

used. The expressed calpains were purified by Ni^{2+}-nitrilotriacetate affinity chromatography, the purification system was from Qiagen (Chatsworth, CA, U.S.A.). For SDS/PAGE, Laemmli's [17] method was used. Gels were stained with Coomassie Brilliant Blue. Protein concentration was determined according to Bradford [18]. All other chemicals were obtained from Sigma.

Construction of mutants

The pET-24-m-80k-$CHis_6$ vector, containing rat m-calpain large subunit (80 kDa) with a C-terminal His-tag, and the pACpET-21k vector, encoding the C-terminal 184 amino acids of m-calpain small subunit as described by Elce et al. [19] were used. The primer sequences used to generate amino acid replacements according to Alexa et al. [12] within pET-24-m-80k-$CHis_6$ are shown in Table 1. All constructions were verified by sequencing.

The 80 kDa large subunit and the 21 kDa truncated small subunit of rat m-calpain were expressed in *E. coli* BL21(DE3) cells and purified as described in [19].

Calpain activity measurements

Enzyme activity was measured with a Jasco FP 777 spectrofluorimeter at excitation/emission wavelengths of 380/460 nm, by continuously recording the increase in fluorescence corresponding to the cleavage of the substrate N-succinyl-Leu-Tyr-7-amino-4-methyl-coumarin (Sigma), in a 3 mm × 3 mm quartz cuvette. The reaction volume was 50 μl, which contained 1 mM fluorescent substrate in a buffer of 50 mM Tris/HCl (pH 7.50), 150 mM NaCl, 1 mM EDTA and 2 mM dithioerythritol. Free-Ca^{2+} concentration was adjusted with EDTA and was checked by a commercial calcium-selective electrode. The reaction was started by the addition of calpain. The calpain progress curves started with a lag-phase, corresponding to calpain activation. The sigmoidal curves were derived to determine maximal activity. Specific activities were calculated at 5 mM Ca^{2+} concentration.

RESULTS AND DISCUSSION

The mutations made in human m-calpain, with their sequential neighbourhood, are presented in Table 2. The sites of mutagenesis were chosen such that their modification, i.e. the replacement of one or more charged residues with neutral one(s), should affect intramolecular salt bridges (cf. Figures 1C and 1D). The measured Ca^{2+}-half-activation concentrations and relative specific activities of the various mutants are given in Table 3. The rationale underlying the evaluation is simple: abolition of an interaction by changing the amino acids involved will lessen the Ca^{2+} demand for activation, if the interaction is to be eliminated during activation of the native enzyme, and vice versa, altering a necessary interaction will increase Ca^{2+} demand.

Mutations in the upper part of the transducer, K505S (Lys^{505} → Ser), K505A/K506S and K505S/K506S all increased the Ca^{2+} demand for activation, suggesting that salt bridges formed by these residues with Asp^{395} and Glu^{393} in the acidic loop in domain III, are to be preserved in the active state. In contrast, mutations D513N/D514N and D513G/D514G/E515G, decreased the Ca^{2+} demand for activation, indicating that the salt bridges between Asp^{513}/Asp^{514} and Arg^{628} in domain IV are to be broken on activation. Mutations towards the lower end of the transducer, E521Q and E522Q, both decreased Ca^{2+} demand, again suggesting the necessity of abolition of the salt bridge between these side chains and Lys^{606} in domain IV on activation.

These findings largely mesh with previous studies concerning the contribution of the extended transducer [12], i.e. the acidic loop in domain III and the transducer connecting domains III and IV, in calpain activation. The involvement of this region has been suggested by the observation that domain III of both μ- and m-calpain is a Ca^{2+}-binding module [13], the possible function of which is to release domain IIb so that it can combine with domain IIa in generating the active, papain-like, catalytic domain [6]. This mechanism has received considerable support since then. Mutagenesis of the acidic loop region neutralizing clusters of negative amino acids increased Ca^{2+} sensitivity, corroborating its Ca^{2+}-binding capacity and involvement in activation [12]. Mutations targeted at the transducer region suggest that this region and the acidic loop form a structural and functional unit that acts in a concerted manner [12].

Fernandez-Montalván et al. [14] studied human μ-calpain in a similar manner by site-directed mutagenesis, monitoring changes in the Ca^{2+} sensitivity of activation. They converted successively seven, out of eight, negatively charged residues in the acidic loop to an alanine residue, one in each mutant: all these mutants exhibited significantly lower $[Ca^{2+}]_{0.5}$ values compared with the wild-type enzyme, in good qualitative agreement with our earlier data [12]. In that previous study, we also modified the protein around residue Glu^{522} by deleting it (M-Δ522) or adding an extra glycine residue (M-Ins522G): these modifications had practically no influence on Ca^{2+} sensitivity, which corroborates our present interpretation. Very recently, Hosfield et al. [20], while characterizing m-calpain forms, found a 42 % decrease in Ca^{2+}-requirement with mutant E517P, which is practically identical with the value measured by us for this mutant ([12], Table 3). The apparently contradictory finding [21] that the replacement E504S at the upper part of the transducer led to a more Ca^{2+}-sensitive enzyme can also be explained within the context of the extended transducer. Since Glu^{504} interacts with the domain IIb, whereas Lys^{505} and Lys^{506} interact with the acidic loop, mutation of

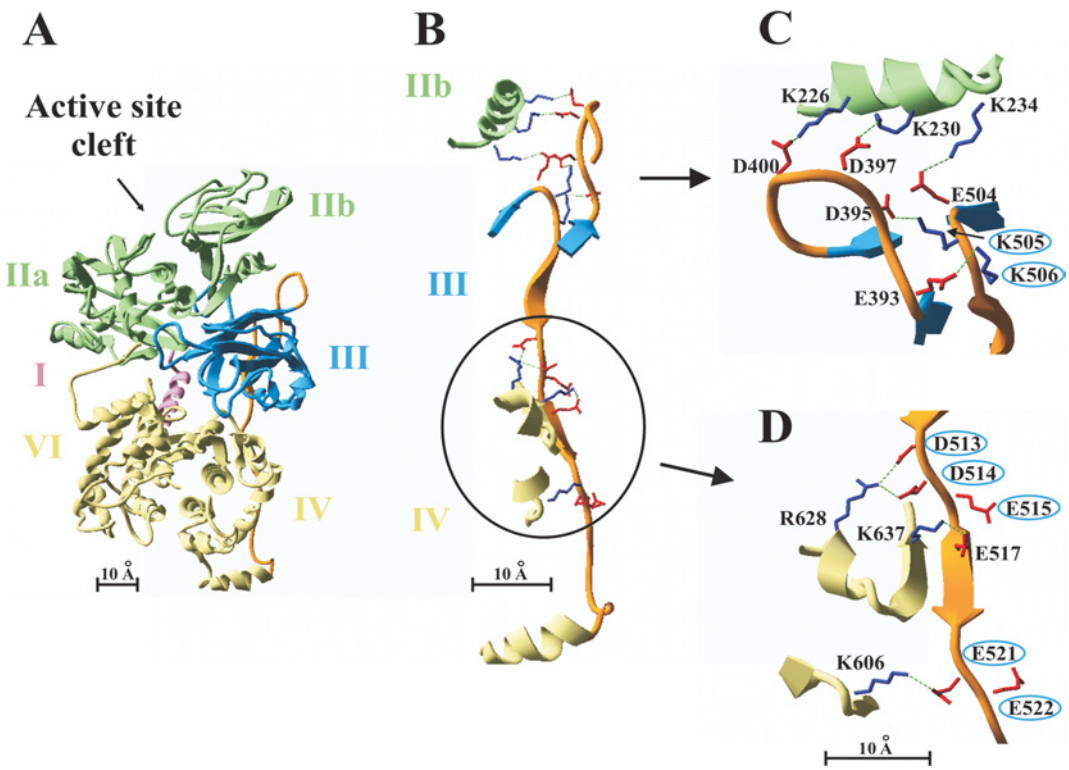

Figure 1 Three-dimensional structure of human m-calpain [6] (A) and an enlargement of parts included in this study: the extended transducer (B) and its sub-regions modified by site-directed mutagenesis (C, D)

Scale bars at the bottom: 10 Å (where 1 Å = 0.1 nm). Domains I–VI are colour-coded as indicated in (**A**). The extended transducer is in orange. In (**C**, **D**), residues mutagenized here are encircled. Other residues are either salt-bridge-forming partners (green broken line) or were involved in our earlier mutagenesis study [12].

Table 3 Kinetic parameters of m-calpain mutants

Enzyme	$[Ca^{2+}]_{1/2}$ (μM)	Relative specific activity (%)
Wild-type	240 ± 30	100
K505S	320 ± 15	147
K505A/K506S	340 ± 26	75
K505S/K506S	370 ± 11	24
D513N/D514N	150 ± 20	143
D513G/D514G/E515G	180 ± 18	149
E521Q/E522Q	158 ± 10	80

Glu[504] may weaken affinity to domain IIb and lie on the path towards activation. Modifying Lys[505] and Lys[506], on the other hand, destroys salt bridges key to the structural integrity of the extended transducer, thus impairing the mechanism of activation and increase the calcium-demand of the enzyme.

The emerging picture (Figure 2) from all these studies is that, during the course of Ca^{2+}-induced activation, the transducer should maintain its connection with the acidic loop, but must break contacts with domain IV. This would result in a downward pulling effect on the acidic loop, relieving the constraint on domain IIb. This transition may be propelled by the outstretched transducer, assuming an energetically more favourable conformation. This mechanistic element is an important component of the subtle structural transition calpain undergoes during activation. As detailed in the Introduction section, the mechanism constitutes sequestering of Ca^{2+} by the calmodulin-like domains IV and VI, with a concomitant small, but critical, conformational change.

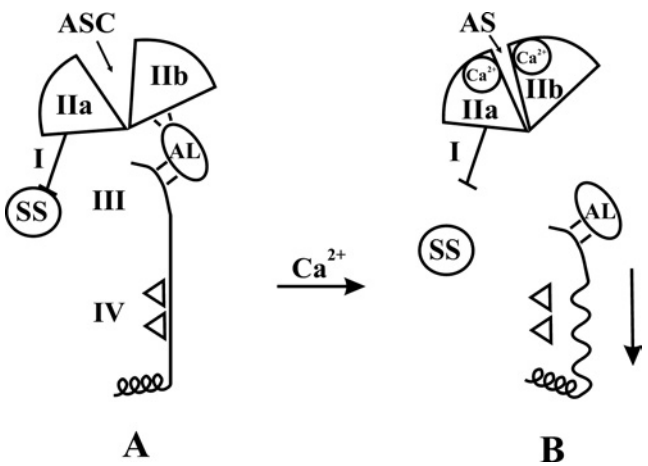

Figure 2 Scheme of relaying structural transition in calpain on activation by Ca^{2+}

In the absence of Ca^{2+} (**A**), the extended transducer involving the acidic loop (AL) is bound at several points to domain IV (triangles). The AL fixes domain IIb by salt bridges, which hinders closure of the active-site cleft (ASC). The AL is also bound to basic residues in the transducer. On the addition of Ca^{2+} (**B**), the two Ca^{2+} sites in domain II make the ASC close, assisted by the removal of the AL due to a pull from the relaxing transducer. Another constraint, imposed on domain IIa by contact with the N-terminal anchor helix, connected to the small subunit (SS), is also abolished by autolysis of the N-terminus and/or dissociation of the small subunit. Among the several bound Ca^{2+} ions, only two that have been located by X-ray analysis in domain II are indicated.

This change is transmitted towards the active site by specific relay systems on both sides of the molecule, i.e. the N-terminal anchor segment and the extended transducer, leading to the release

of the catalytic domain from the structural constraints imposed in the inactive enzyme. All these culminate in the Ca^{2+} binding of domain II and the closure of the active-site cleft. In all its details, our current observations fit into this general and ever-more detailed activation picture of typical calpains. By all means, this is an outstanding example of how multiple activator sites may co-operate in channelling their effect through the protein fabric to the active site of an enzyme.

This work was supported by OTKA (Hungarian Scientific Research Fund, Budapest, Hungary) grants T 34255 and OTKA TS 040723 to P.F. Support from the Bolyai János Scholarship is acknowledged by A. A. and P. T.

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Received 19 November 2004/29 November 2004; accepted 30 November 2004
Published as BJ Immediate Publication 30 November 2004, DOI 10.1042/BJ20041935