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MUTATION OF AN IQ-MOTIF: EVIDENCE FOR AN EXOSITE IN CALPAIN-2?

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ABSTRACT:

These studies aimed to test the hypothesis that calcium regulates calpain activity by a mechanism analogous to that used by Ca^{2+}-calmodulin (CaM) to regulate its target enzymes. Site directed mutagenesis of a putative IQ motif, $IQ_{413}xxxR_{417}$ generated catalytic subunits modified at both, or each, of the key residues of the motif to yield ISxxxQ, ISxxxR, and IQxxxQ. Each of the mutant catalytic subunits was co-expressed with a truncated (21k) small subunit in *E. coli*. The heterodimeric enzymes were purified by standard methods suggesting each was properly folded. Electrophoresis under native conditions, in the absence of calcium, showed no differences between wt and enzyme mutated at $R_{417}Q$. Each of these enzymes was also stable to incubation at 45 ºC. In contrast enzymes containing $Q_{413}S$ were resolved into multiple bands on native gels and were not stable at 45 ºC. Two calcium dependent activities were assessed: casein hydrolysis and autoproteolysis. Each of the mutants retained significant autolytic activity but lost much of their caseinolytic activity. We propose two interpretations of the results based in part on the calcium-free structures determined for calpain-2 (Hosfield et al , 1999 EMBO J. 18, 6880 and Strobl et al 2000, PNAS 97, 588). The ability of the enzymes to autoproteolyze suggests that the conformational change required for alignment of the catalytic residues is unimpaired in the mutants. Impaired casein hydrolysis may result from 1) inability to release product after cleavage or 2) failure to form a required substrate binding exosite. The latter concept is consistent with many previously known calpain attributes. Exosites provide a mechanism for achieving high substrate specificity amongst enzymes that share conserved active sites, such as those of the thrombin family and perhaps the calpain family. Confirmation and definition of such exosites will be important as they could provide targets for design of isoform selective inhibitors.

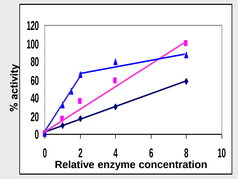
CHARACTERIZATION OF IQ-MOTIF MUTANTS IN THE PRESENCE OF CALCIUM

A. CASEIN HYDROLYSIS

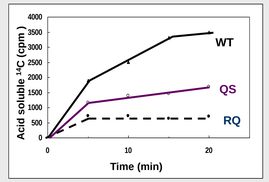
A1. SPECIFIC ACTIVITY as % unmutated calpain-2

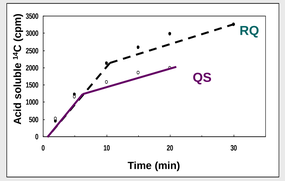
$Q_{413}SR_{417}Q$	$Q_{413}S$	$R_{417}Q$
< 0.2	< 3	< 15

A2. ACTIVITY PROPORTIONAL TO ENZYME CONCENTRATION but NOT LINEAR with TIME



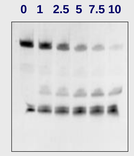
WT(♦) 5 -80 ng, $R_{417}Q$ (■) 50 -400 ng, $Q_{413}S$ (♦); 100% activities ranged from 1-2k cpm acid soluble ^{14}C



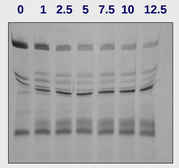


Assays of casein hydrolysis were conducted at 25 ºC (left) or 4 ºC (right)

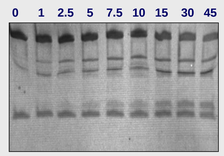
B. AUTOPROTEOLYSIS



Wild type



$R_{417}Q$



$Q_{413}S+R_{417}Q$

Autoproteolysis was measured by densitometric loss of the 80k band. Initial rates of autoproteolysis were approximately equivalent for wt and $R_{417}Q$. $Q_{413}S+R_{417}Q$ was approximately 4 fold slower than wt. Rates were typically biphasic for mutants but not for wt.

IN THE PRESENCE OF CALCIUM

- Caseinolytic activity is poor.
- Autoproteolysis occurs at normal or only slightly reduced rates.

Note: Precedence exists for a properly folded calpain ($K_{230}E$ or $K_{234}E$) to display poor caseinolytic activity. (Hosfield et al 2001, J. Biol Chem. 276, 7404-7407)

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We suggest two alternative interpretations of the phenotype of these mutant calpains:

1. Substrate binding is unimpaired but release of product is hampered. OR

2. An exosite required for binding substrate fails to form when the IQ-motif is mutated.

Each of these possibilities presents future opportunities:

Impaired substrate release may allow design of substrate-'trap' mutants useful for identifying *in vivo* targets.

Well defined exosites would allow potential design of isoform specific inhibitors.

While further studies are required to determine which, if either of these alternatives is correct, several facts are consistent with the possibility of an exosite.

- Calpains' preference for protein v. peptide substrates
- Lack of specificity for residues immediately adjacent to the cleavage site
- Interactions between calpain and the conserved domains of calpastatin map to regions distant from the catalytic site
- The environment of the IQ motif changes in the presence of calcium (Moldoveanu et al 2001, BBA)

CHARACTERIZATION OF CALPAIN HETERODIMERS WITH MUTATIONS IN IQ MOTIFS: IN THE ABSENCE OF CALCIUM

1A

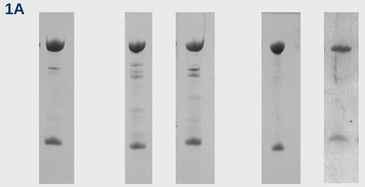


FIGURE 1A. SDS-PAGE

Recombinant calpains were expressed in *E. coli* and purified by: ion exchange, reactive red agarose and Ni-NTA agarose. Figure 1A shows coomassie blue stained SDS-PAGE.

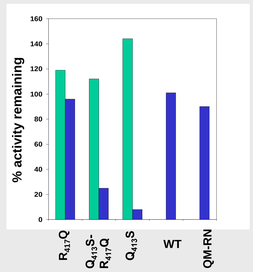
4 μg of each protein was loaded.

FIGURE 1B: NON DENATURING PAGE

Electrophoresis was carried out at 4º C using two different non-denaturing buffer systems: alkaline Tris-glycine (LEFT panel) or neutral Tris-cacodylate (RIGHT panel). Both buffers contained EDTA and reducing agents.

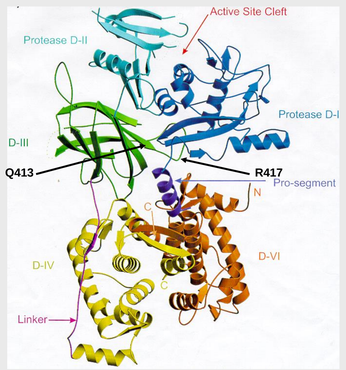
FIGURE 2 STABILITY OF MUTANT CALPAINS

Each enzyme was incubated at 30 ºC (green) or 45 ºC (blue) for 30 min prior to dilution and assay of casein hydrolysis. Data are shown as % activity remaining relative to controls that were not exposed to temperatures higher than 4 ºC.



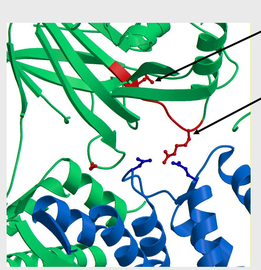
IN THE ABSENCE OF CALCIUM

- Heterodimers are synthesized and assembled as soluble proteins.
- The mutation $Q_{413}S$ appears to favor formation of some higher molecular weight complexes and increased susceptibility to denaturation.
- $R_{417}Q$ is indistinguishable from unmutated enzyme.



The IQ-motif in context :

- lies at interface between D-1 and D-VI
- is conserved in calpains containing EF-hands (calpains 1-3, 9, Drosophila A, B, and Schistosoma mansoni) but not in tra-3, CAPN 5, 6 or 8
- residues 407 and 420 become protease accessible in the presence of calcium. (Moldoveanu et al BBA '01)



Potential interactions for Q413 and R417:

R417 side chain flexible but within reach of E30 (in EF1) or D70 (in EF-2) of D-VI

Thanks to Dr. Chris Hosfield (formerly Queen's U.) for providing the ribbon diagrams in these figures.

FUTURE CHALLENGES:

Can we explain persistence of autoproteolysis that is likely to be intermolecular when hydrolysis of other substrates is severely impaired?

Could aggregation or formation of multimers contribute to that catalytic phenotype?

What substrate(s) will best allow confirmation and characterization of an exosite?

A well expressed, relatively small, cloned protein or domain with high affinity (ability to compete for casein?) would be ideal

Can C105S - R417Q provide a high affinity 'substrate trap' to isolate physiological substrates?