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Calcium-dependent proteolysis and isopeptide bond formation: Calpains and transglutaminases

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Abstract - The mechanisms of action and physiological functions of two calcium-activated enzyme families, the calpains and transglutaminases, are discussed:

1. Both enzymes work via a covalent acylenzyme intermediate. Calpains, as other papain-like proteases, have a Cys and His residue in the active site, which in the ground state tend to form a thiolate-imidazolium ion pair. In the active site of tranglutaminases only a Cys residue is usually considered, although data point to the participation of a His. Sequence analysis of transglutaminases reveals two conserved segments with a His in each, either of which may be part of the active site.
2. The physiological functions of the two enzyme families are reviewed. Special attention is given to neuromodulatory (plastic) changes in the nervous system. Two cases are highlighted: a/ The R-subunit of cAMP-dependent protein kinase undergoes limited proteolysis in Drosophila brain, which prolongs kinase action and thus it seems to be part of an intermediate memory process. b/ The covalent crosslinking of synaptic structures by transglutaminase may contribute to lasting information storage, as suggested by the dramatic increase in the amount of "isopeptide" bond in rat hippocampal slices during long-term potentiation (LTP), an experimental model of long-term memory.

INTRODUCTION

Among the effectors of the multifarious regulatory roles of calcium ions are the enzymes that cleave and synthesize peptide bonds. These are the enzyme families of calpains and transglutaminases, respectively. Both types of enzymes perform practically irreversible modification on proteins *in vivo*, and the two functions are, in a sense, of opposite character: while calpain degrades proteins, as a rule in a limited proteolytic process, transglutaminases build up large covalent protein assemblies by introducing crosslinks. Both enzyme families are ubiquitous in animal cells and are thought to participate in a variety of life processes. Before assessing these we consider some structural and mechanistic features of these enzymes pointing out established and likely similarities. Indeed, there seems to be remarkable parallelism between these enzymes both at the level of the chemistry of the catalyzed reaction and, in a quite different context, in their ability to subserve the maintenance of long-term cellular states ranging from skin cornification to memory.

STRUCTURAL–FUNCTIONAL PROPERTIES OF CALPAINS AND TRANSGLUTAMINASES

Subunit and domain structure

Calpain (calcium-activated neutral thiol protease, EC 3.4.22.17) occurs in mammals as two isoenzymes, calpain I and II, characterized in terms of their activation by uM and mM calcium concentrations, respectively (ref.1). Calpains have a specific high Mr, heat-stable protein inhibitor called calpastatin (ref.2) and a low Mr activator protein (ref.3). These components constitute a versatile calcium-dependent cytoplasmic proteolytic system.
Mammalian calpains are composed of a large subunit (Mr=80 kD) and a small subunit (Mr=30 kD); the catalytic activity resides in the large subunit, which consists of four domains, I through IV, from the N-terminus. The functions of domains I and III are unknown. Domain II corresponds to a cysteine protease (cf. below) and domain IV is a calmodulin-like unit with four calcium-binding EF-hand structures. The small subunit has a Gly-rich N-terminal segment surmised to mediate membrane adherence, and a C-terminal calmodulin-like domain, highly homologous to domain IV in the large subunit.

Transglutaminases are of several types (ref.4, 5). Among the extracellular transglutaminases the plasma enzyme, factor XIII in blood coagulation, is known best. This enzyme is a heterotetramer composed of two a (catalytic) subunits of Mr=75 kD, and two b (non-catalytic) subunits of Mr=80 kD. Upon calcium binding the holoenzyme dissociates and the a subunits are modified by thrombin to become the enzymatically active a' species. Intracellular (tissue) transglutaminases are monomeric and are not proenzymes. Their polypeptide chain length varies considerably, as shown by the following sequenced examples, according to tissue origin (no. of residues): rabbit epidermal transglutaminase, 381 (ref.6); guinea pig liver transglutaminase, 691 (ref.7), human and rat epidermal (keratinocyte) transglutaminase, 788-817 (ref.8,9). These transglutaminases have putative calcium-binding site(s) predicted from their amino acid sequence, but these do not cluster into a calmodulin-like domain as with calpains.

Reactions catalyzed by calpain and transglutaminase

Calpain catalyzes, in the presence of calcium, the hydrolysis of peptide bonds in proteins. Its sequence homology with other cysteine proteases suggests that the mechanism established for papain by Polgár (ref.10) also applies to it (Fig.1). According to this mechanism, in the active site a thiolate-imidazolium ion pair, constituted by Cys-25 and His-159 in the case of papain, makes a nucleophilic attack on the carbonyl C-atom of a peptide bond (or of an ester bond with ester substrates) forming a tetrahedral intermadiate (THI). The THI is converted to a covalent intermediate acyl-enzyme via general acid catalysis by the protonated imidazole group. In the deacylation process the attacking nucleophile is a water molecule which, assisted through general base catalysis by the unprotonated imidazole group, forms the THI, which in turn decomposes regenerating the free enzyme.The characteristic feature of cysteine protease, and thus probably calpain, catalysis is the existence of an intimate thiolate-imidazolium ion pair in the ground state of the enzyme. This ion pair was clearly demonstrated in the case of papain by the pH dependence of alkylation kinetics of the active site SH (ref.11) and also spectrophotometrically (ref.12).

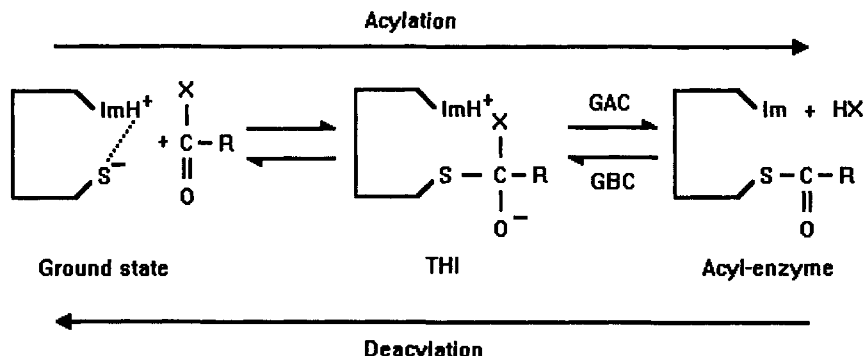

Fig. 1. Mechanism of action of cysteine proteases [modified from Polgár (ref.10)]. X denotes OR' or NHR' in acylation, OH in deacylation. GAC and GBC stand for general acid and general base catalysis, respectively. Im=imidazole group, THI=tetrahedral intermediate.

Transglutaminase (protein-glutamine:-amine-γ-glutamyl transferase, EC 2.3.2.13) catalyzes calcium-dependent acyl-transfer reaction between protein-bound glutaminyl residues and primary amines (for a recent review see ref.5). The specificity for protein-bound glutamine is very strict, while the enzyme accepts a variety of primary amines as second substrate. Therefore transglutaminase may produce two types of product: 1/ with low Mr amines proteins get modified by the attachment of these groups through glutamyl side chains; 2/ if the amine is the ε-NH2 group of a protein-bound Lys, then an isopeptide, ε-(γ-glutamyl)lysine, bond crosslinking the two proteins is formed. It will be shown below that this interprotein crosslink may be a mechanistic device in a great number of life processes.

Active site homologies of calpain and transglutaminase

Cysteine proteases have a highly conserved amino acid sequence around the active site Cys residue. As seen in Fig.2, the observed similarity score, based on the log-odds score table of Dayhoff (ref.13), for a decapeptide between calpain and six other cysteine proteases ranges from 112 to 117, while the expected score for random decapeptides would be 72. Interestingly, in the same comparison transglutaminases are slightly more similar to calpain (score: 124 and 125) than are the other proteases. Clearly, the same protein module must have been used in evolution to construct active site regions in these enzymes.

Enzyme	Sequence	Similarity score
Human Calpain I,II	LGDCWLLAAI	--
Guinea Pig Liver TGase	YGQCWVFAAV	125
Factor XIIIa	YGQCWVFAGV	124
Epidermal TGase	YGQCWVFAGV	124
Actinidin	CGGCWAFSAI	117
Papain and Protease Ω	CGSCWAFSAV	115
Chymopapain	CGSCWAFSTI	115
Cathepsin H	CGSCWTFSTT	113
Cathepsin B	CGSCWAFGAF	112

Fig. 2. Sequence homology of an active site decapeptide in cysteine proteases and transglutaminases. Similarity score was calculated according to Dayhoff (ref.13). Black triangles indicate the acylenzyme-forming Cys residue.

In papain-like enzymes, however, there is also a His residue in the active site, as discussed above. In calpain, along with Cys-108, the His-265 residue is involved (ref.1). Nevertheless, in recent considerations on transglutaminases (ref.5) the contribution of a His residue to the catalytic mechanism is not invoked. This is somewhat striking, because earlier studies on the pH-dependence of alkylation with iodoacetamide (ref.14) revealed an apparent pKa=6.0 for the active site SH-group, whereas the pH-profile of the steady-state velocity of the methanolysis of [dansyl-(N-methyl-β-alanyl)]thiocholine iodide by factor XIIIa displayed a pKa=6.7 (ref.15). Both pKa values are well below that of a free SH-group, and indeed in these early studies the participation of a histidine side chain was raised (ref.15). Furthermore, this seems reasonable because the imidazole moiety assists not only the nucleophilic attack leading to the acylenzyme, but also the decomposition of the acylenzyme through general base catalysis. Such facilitation may be essential for a reasonable catalytic efficiency (turnover). Therefore we suggest that the scheme of Fig.1 also applies to transglutaminase with the modification that X denotes NH2 in the acylation step and RNH in the deacylation step.

If a His residue is crucial for transglutaminase activity, it should be in a conserved segment of the enzyme. Analyzing the available amino acid sequences we found only two His residues, out of 12 to 20, that occurred in a conservative environment (Fig.3).

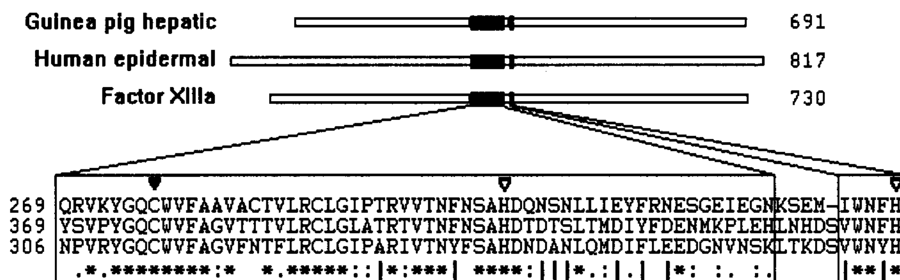

Fig. 3. Two conserved His residues in transglutaminases. The three different sequences are drawn to scale (total number of amino acid residues indicated), aligned at the active site region, which is blown up below. The black triangle marks the active Cys, the hollow triangles denote the two conserved His residues which may play a role in catalysis. Homology symbols: *, identical amino acids; |, strongly; :, medium; ., weakly conserved amino acids.

These His residues are 28 and 57-58 residues toward the C-terminus from the active site Cys. Although this distance is markedly shorter than that found in calpain (157 residues) and other cysteine proteases, it may be long enough for an appropriate fold in the steric structure. Site-directed mutagenesis of these histidines, as well as the unravelling of the 3D structure of tranglutaminase, will eventually test this hypothesis. Human erythrocyte band 4.2 protein was found to be homologous to

transglutaminases (ref.16), in particular around the active site Cys residue, which itself was replaced by an alanine; this protein is devoid of any transglutaminase activity. It is interesting to mention that in band 4.2 protein both the above histidines are replaced by glutamines.

PHYSIOLOGICAL FUNCTIONS OF CALPAINS AND TRANSGLUTAMINASES

Both enzymes mediate calcium effects inside and outside of cells. For activation they do not need the ubiquitous calcium-mediator protein calmodulin, which when complexed with calcium activates many enzymes of central regulatory functions: protein kinase, phosphodiesterase, etc. Calpain and transglutaminase have the calcium-binding sites built in their primary structure. The modifications these enzymes make in proteins are practically irreversible; although the "isodipeptide" ε-(γ-glutamyl)lysine can be cleaved enzymatically in vitro (ref.17), this reaction does not work with proteins in vivo. Since the modified proteins remain such until replaced by protein turnover, one expects that these modifications serve longer-term goals than other, readily reversible modifications, such as phosphorylation.

Calpains

Calpains are thought to be involved in a great variety of cellular processes through the limited proteolysis of substrate proteins. The substrate specificity of calpains is not clear: the residues around the peptide bond cleaved were found to vary with different proteins and peptides (ref.18). It appears that calpains recognize some higher order structure in proteins, possibly by one of the domains other than the cystein protease domain. The PEST-sequences have been proposed to serve as calpain recognition motifs (ref.19), but more evidence is needed to substantiate this claim. [PEST-sequences are short segments in proteins rich in Pro, Asp, Glu, Ser and Thr, and flanked by basic residues, and are regarded as signals for proteolytic attack (ref.20)]. Microtubule-associated proteins (MAPs) are the richest in PEST-sequences among the proteins analyzed so far (ref.21) and most of them are very sensitive to calpain attack.

The proteins that so far were found to be cleaved by calpain, at least in vitro, fall into several categories. Although calpain acts on some peptides (e.g. enkephalins, somatostatin, etc.), its preferred substrates are proteins. These macromolecular substrates are enzymes, receptors, growth and blood clotting factors, cytoskeletal proteins, protease inhibitors (e.g. its own inhibitor, calpastatin), and some other proteins. Limited calpain proteolysis often results in the activation of the enzymes, for example by abolishing the requirement for an activator. Prolonged exposure to calpain, however, destroys most of these proteins along with their activity so that activation is, as a rule, transient. Detailed lists of substrates can be found elsewhere (ref.19,33). The physiological and pathological processes in which calpains have been implicated are also numerous: regulation in signal transduction, muscle protein degradation (muscular dystrophy), cell fusion, aging, cataract formation, Wallerian degeneration (due to neurofilament degradation), etc. The ubiquity of calpains in mammalian cells suggests that, in addition to tissue-specific functions, they play some general "housekeeping" role, such as cytoplasmic protein catabolism. If so, then calpain is involved, directly or indirectly, in all life processes insofar as without calpains cells cannot exist. In fact, the failure to establish cell lines deficient in calpain supports this tenet.

The connection of calpains with such higher order functions as learning and memory is particularly intriguing. Long-term synaptic changes, i.e. lasting modulations of transmission between nerve cells, are likely to be part of the mechanism underlying long-term information storage. These involve the restructuring and stabilization of cytoskeletal assemblies, also accommodating various signal-transducing proteins, in the nerve cell processes, mainly dendrites (ref.22,23). Since these changes are triggered by enhanced firing in the neurons involved, which raises intracellular calcium concentration, calcium-activated enzymes may be instrumental. It has been suggested that postsynaptic restructuring is initiated by the partial breakdown of dendritic cytoskeleton by calpain (ref.22). Another route through which calpain may act is the modification of enzymes, e.g. protein kinases, prolonging their activity. In the fruit fly Drosophila melanogaster cAMP-dependent phosphorylation is pivotal in olfactory associative learning (ref.24). Part of the intermediate-term memory seems to reside in the sustainment of the cAMP-dependent protein kinase (PKA) activity after a transient rise in cAMP level. This can be achieved by the limited proteolysis of the regulatory (R) subunit of PKA by calpain (ref.25), which also occurs in Drosophila (ref.26). The model we proposed (ref.27) for the molecular mechanism of this learning process consists in the convergence of two stimuli on PKA: cAMP dissociates and activates PKA, while calcium activates calpain, which in turn produces a truncated Rp subunit. Since the avidity of Rp to recombine with the catalytic C subunit is markedly diminished as compared with unmodified R, the PKA activity will be substantially prolonged, thereby maintaining an altered phosphorylation state of specific substrate proteins and by the same token the altered behaviour.

Transglutaminases

Extracellular transglutaminases have clear-cut biological functions (ref.4). The prototype blood coagulation factor XIII is essentail for fibrin stabilization; its hereditary deficiency leads to life-threatening hemorrhages. The formation of copulation plug by transglutaminase from the seminal fluid of rodents is another plausible function. As for intracellular transglutaminases, the production of an insoluble "cornified" envelope in epidermal keratinocytes (ref.28) serves the mechanical stability of skin. The rigidification of erythrocyte membrane owing to transglutaminase-catalyzed crosslinking of submembrane protein network may be a general paradigm for terminal differentiation, aging and dying of cells, as in programmed cell death (apoptosis) (ref.28). In the eye lens this may lead to cataract formation and in the brain transglutaminase has been invoked in the formation of paired helical filaments, hallmarks of Alzheimer's disease (ref.4).

The covalent crosslinking of proteins in nerve cells, however, need not only lead to pathological processes. Transglutaminase levels undergo marked changes during neuronal maturation (ref.30). The stabilization of synaptic structures after plastic changes discussed in connection with calpain may conceivably be served by crosslinking. To test this hypothesis, we induced long-term potentiation (LTP) in rat hippocampal slices _in vitro_ (ref.31). LTP is a vigorously investigated electrophysiological model of long-term memory: strong presynaptic stimulation or mild combined pre- and postsynaptic stimulation of CA1 pyramidal cells brings about long-lasting hyperreactivity of these cells (ref.32). LTP is induced by a rise in post-synaptic calcium concentration, through the activation of various calcium-dependent enzymes. In our studies the induction of LTP was followed by a dramatic increase in the amount of ε-(γ-glutamyl)lysine in the proteolytic hydrolysate of the hippocampal slice (ref.31). This can only be explained by an elevation of transglutaminase activity in the sample tissue. Further experiments are to decide whether tranglutaminase action is indeed essential for the cellular machinery of LTP. At any rate, our observation encourages the scrutinizing of tranglutaminase-induced protein crosslinking in various other long-term neuromodulatory processes.

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