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The proteasome/multicatalytic–multifunctional proteinase

In vivo function in the ubiquitin-dependent N-end rule pathway of protein degradation in eukaryotes

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Proteinase yscE, the proteasome/multicatalytic–multifunctional proteinase of yeast had been shown to function in stress response and in the degradation of ubiquitinated proteins [(1991) EMBO J. 10, 555–562]. A well-defined set of proteins degraded via ubiquitin-mediated proteolysis are the substrates of the N-end rule pathway [(1986) Science 234, 179–186; (1989) Science 243, 1576–1583]. We show that mutants defective in the chymotryptic activity of proteinase yscE fail to degrade substrates of the N-end rule pathway. This gives further proof of the proteasome being a central catalyst in ubiquitin-mediated proteolysis.

Proteinase yscE; Proteasome/multicatalytic–multifunctional proteinase; Ubiquitin; N-end rule; Proteolysis; Yeast

1. INTRODUCTION

The proteasome/multicatalytic–multifunctional proteinase belongs to a class of protein complexes, which can be found in all eukaryotic cells and which has been conserved during evolution from yeast to man [1–7]. The protein particle of about 700 kDa mass consisting of at least 12 different subunits in the molecular mass range of between 20 and 38 kDa has been extensively studied in vitro (for reviews, see [6,7]), but until recently [8] no in vivo function could be assigned to the protein complex. Speculative functions for the particle ranged from controlling mRNA-activity [9] to protein maturation and degradation (for reviews, see [6,7]). Three proteolytic activities could be assigned to the proteasome. These activities have been called 'chymotrypsin-like', 'trypsin-like' and 'peptidylglutamyl-peptide hydrolyzing' activity (for reviews, see [6,7]). The yeast *Saccharomyces cerevisiae* has turned out to be an excellent model organism to study eukaryotic cell function as this organism is easily accessible to biochemical, genetic and molecular biological manipulation. As such, yeast has given central insights into the function of proteolysis in eukaryotic cells (for reviews, see [10–13]). We had detected, purified and characterized the yeast proteasome/multicatalytic–multifunctional proteinase, which we had named proteinase yscE [14]. Mutations in the chymotryptic activity of proteinase yscE had for the first time given in vivo evidence that the proteasome is involved in intracellular protein degradation and stress response [8]. One degradation pathway which is severely affected by a defective chymotryptic activity of proteinase yscE is ubiquitin-mediated proteolysis, as the bulk of ubiquitinated proteins accumulate intracellularly in proteinase yscE mutant cells [8]. Specific substrates, which are known to be degraded via ubiquitin-mediated proteolysis consist of substrates of the N-end rule pathway [15–18]. Here we show that the yeast proteasome is linked to degradation of substrates of the N-end rule pathway.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

The inhibitors pepstatin A, chymostatin, antipain, leupeptin and aprotinin were obtained from the Peptide Institute (Osaka, Japan). The monoclonal antibodies recognizing β-galactosidase were purchased from Boehringer Mannheim (Mannheim, Germany) and Promega (Heidelberg, Germany). [^{35}S]methionine was obtained from Amersham (Braunschweig, Germany). Yeast nitrogen base without amino acids was purchased from Difco (Detroit, USA). All other chemicals were obtained from Pharmacia (Freiburg, Germany), Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany) and Serva (Heidelberg, Germany).

2.2. *Media*

YPD medium (2% glucose, 1% yeast extract, 2% peptone) and complete minimal (CM) dropout medium were prepared according to Ausubel et al. [19]. CM dropout medium contained 0.67% yeast nitrogen base without amino acids and 2% galactose supplemented with adenine and amino acids. The [^{35}S]methionine labeling medium used, consisted of CM dropout medium without uracil and methionine.

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2.3. *Strains*

Yeast strains used were: BR1 (*MATa ura3 his3-11,15 GAL*$^+$); BR2 (*MATa pre1-1 ura3 GAL*$^+$); BR3 (*MATa pre2-2 ura3 his3-11,15 leu2-3,112 GAL*$^+$); BR4 (*MATα pre1-1 pre2-2 ura3 his3-11,15 leu2-3,112 GAL*$^+$)

Strains BR1 and BR2 were obtained by mating strain 95/2-3D/15D (*MATα pre1-1 ura3-5 his3-11,15 leu2-3,112*) with strain L3906 (*MATa trp1-1 ura3-52 GAL*$^+$) followed by isolation of diploids and subsequent sporulation.

Strain BR3 was obtained by mating strain 119/3-9D (*MATα pre2-2 ura3-5 his3-11,15 leu2-3,112*) with strain L3906 followed by isolation of diploids and subsequent sporulation.

Similarly strain BR4 was obtained from a cross of strain BR2 with strain 119/3-9D. *E. coli* strain MC 1061 was used as host of plasmids.

2.4. *Molecular biological techniques and plasmids*

Isolation, purification and analysis of DNA were carried out according to standard procedures [20]. For transformation a protocol modified according to [8] was followed. The yeast strains were transformed with the 2μ-based plasmids pUB23 expressing ubiquitin-X-β-galactosidase fusion proteins under control of the galactose inducible *GAL1* promotor [15,18].

2.5. *Pulse-chase experiments and immunoblotting*

Labeling of cells, preparation of cell extracts, immunoprecipitation of β-galactosidase antigenic material, SDS-PAGE and fluorography were essentially done as described by Bartel et al. [21]. Prior to labeling, cells were grown 24 h at 30°C in CM medium. After centrifugation cells were diluted into fresh CM medium yielding an $OD_{578\,nm}$ of 0.5 and incubated for 3 h at 30°C. SDS-PAGE was done in a 7.5% gel. Immunoblotting was done according to Towbin et al. [22].

2.6. *Preparation of extracts and enzyme assay*

Wild type and mutant strains carrying the respective ubiquitin-X-β-galactosidase expressing plasmid were grown in CM dropout medium containing 2% galactose for 24 h at 30°C. Cells were harvested by centrifugation and extracts were prepared in Eppendorf tubes using glass beads [8]. The buffer was 0.1 M potassium phosphate pH 7.0 containing chymostatin (20 μg/ml), pepstatin (20 μg/ml) and 4-hydroxymercuribenzoate (5 mM). Enzymatic activity of β-galactosidase in crude extracts was measured using *o*-nitro-phenyl-β-D-galactoside, as outlined in [19]. The amount of protein was determined by the method of Lowry et al. [23].

3. RESULTS

We had isolated mutants defective in the chymotryptic activity of proteinase yscE. The mutants fell into two complementation groups *pre1* and *pre2* [8]. The wild-type genes (*PRE1* and *PRE2*) were isolated and sequenced and shown to encode subunits of proteinase yscE of 22.6 kDa and 31.6 kDa, respectively [8,24]. Mutations in either of the two subunits of the enzyme resulted in a defective stress response and led to an accumulation of ubiquitinated proteins [8,24]. Deletion of either of the two subunits leads to cell death [8,24].

To test the function of proteinase yscE in distinct proteolytic pathways known to be dependent on ubiquitin, we chose the N-end rule pathway. Using well-defined engineered fusion proteins it was uncovered that the amino terminus of the fusion protein acts as a primary signal determining its half-life [15]. As a secondary signal for degradation to occur the posttranslational coupling of a multiubiquitin chain onto the protein is necessary [17,18]. The engineered proteins used were ubiquitin-X-β-galactosidase fusion proteins (Ub-X-βgal), where 'X' stands for any amino acid residue. In the yeast cell, ubiquitin C-terminal hydrolases rapidly remove ubiquitin from the fusion protein, exposing the amino terminal residue 'X' of the remaining part of the protein [15]. An exception is ubiquitin-proline-β-galactosidase (Ub-Pro-βgal), which is only very slowly cleaved by the hydrolase [15]. β-galactosidase proteins carrying N-terminal amino acids which destine the protein to rapid degradation (Arg, Leu, Tyr) undergo attachment of a multiubiquitin chain onto a specific lysine residue of the substrate [15,17,18]. This ubiquitination reaction is dependent on the substrate recognition pro-

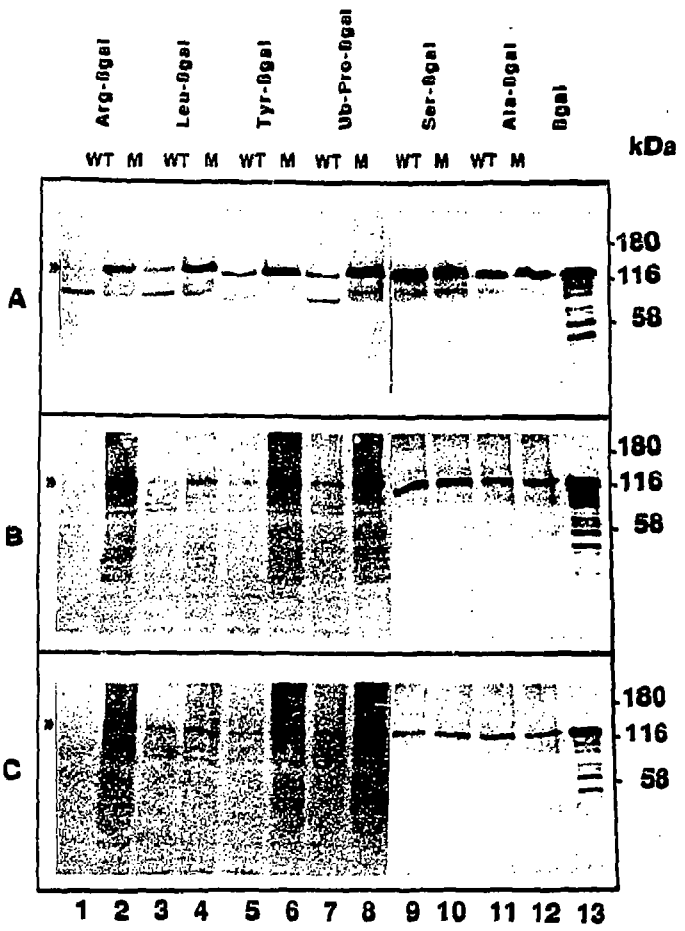

Fig. 1. Steady-state levels of X-βgal test proteins in wild-type and mutant cells defective in chymotryptic activity of proteinase yscE. Cells were grown and disintegrated as indicated in Section 2. (A) Wild-type BR1, lanes 1,3,5,7,9,11; *pre1-1* mutant BR2, lanes 2,4,6,8,10,12. (B) Wild-type BR1, lanes 1,3,5,7,9,11; *pre2-2* mutant BR3, lanes 2,4,6,8,10,12. (C) Wild-type BR1, lanes 1,3,5,7,9,11; *pre1-1 pre2-2* double mutant BR4, lanes 2,4,6,8,10,12. X-βgal proteins tested: Arg-βgal, lanes 1 and 2; Leu-βgal, lanes 3 and 4; Tyr-βgal, lanes 5 and 6; Ub-Pro-βgal, lanes 7 and 8; Ser-βgal, lanes 9 and 10; Ala-βgal, lanes 11 and 12; lane 13, β-gal. Molecular mass markers were: α_2-macroglobulin, M_r = 180 kDa; β-galactosidase, M_r = 116 kDa; fructose-6-phosphate kinase, M_r = 84 kDa; pyruvate kinase, M_r = 58 kDa; fumarase, M_r = 48.5 kDa.

tein Ubr1 (21) and the Ubc2 ubiquitin conjugating enzyme [25–27]. Ub-X-βgal fusion proteins which are not deubiquitinated because of the nature of 'X' (proline) are short-lived and undergo a multi-ubiquitination reaction at Lys^{48} of the N-terminal ubiquitin [18]. This reaction is dependent on the ubiquitin conjugating enzymes Ubc4 and Ubc5 [18].

To test the involvement of proteinase yscE in the ubiquitin-dependent N-end rule pathway we analyzed the stability of short-lived β-galactosidase fusion proteins in *pre1-1* (Table I, Fig. 1) and *pre2-2* (Table I, Figs. 1 and 3) mutant cells as well as in double mutants (*pre1-1 pre2-2*) (Table I, Figs. 1 and 3) defective in both subunits of proteinase yscE, which in their intact form confer the chymotryptic activity to the enzyme. Both mutations consist of an amino acid exchange each in the respective subunit: *pre1-1* leads to a change of Ser^{142} to Phe^{142}, *pre2-2* to a change of Ala^{124} to Val^{124} [24].

Cells were transformed with the plasmids expressing the different engineered Ub-X-βgal proteins. At first we measured β-galactosidase (βgal) activities and the protein levels of Arg-βgal ($t_{1/2}$~2 min), Leu-βgal ($t_{1/2}$~3 min), Tyr-βgal ($t_{1/2}$~10 min), Ub-Pro-βgal ($t_{1/2}$~7 min), Ser-βgal and Ala-βgal ($t_{1/2} \geq 20$ h) [15] in extracts of wild-type and mutant cells. These measurements were expected to result in steady-state activity and protein levels of the metabolically differently stable proteins. As can be seen from Table I only very low specific βgal activities can be found in wild-type cells of the metabolically highly unstable fusion proteins Arg-βgal, Leu-βgal, Tyr-βgal and Ub-Pro-βgal while considerably higher activities are found for the metabolically stable fusion proteins Ser-βgal and Ala-βgal. In the proteinase yscE mutants carrying either the *pre1-1* or the *pre2-2* mutation or being defective in both subunits (*pre1-1, pre2-2*) considerably higher βgal activities were found for the metabolically unstable fusion proteins as compared to wild-type (Table I). Increase of βgal activity in *pre1-1* mutant cells varied from 9-fold for Arg-βgal (the most unstable protein) to three-fold for Ub-Pro-βgal, a less unstable protein. In contrast, no significant alteration in βgal activity was found for the metabolically rather stable proteins Ser-βgal and Ala-βgal (Table I). Increase in βgal activity as compared to wild-type of the metabolically unstable X-βgal proteins was even somewhat higher in *pre2-2* mutant cells and highest in *pre1-1 pre2-2* double

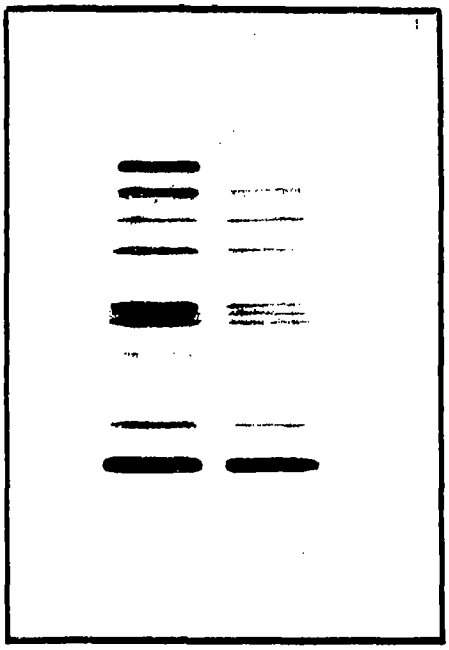

Fig. 2. Purified β-galactosidase competes with a distinct protein band of Ala-βgal-expressing cells. Strain BR3, transformed with the Ala-βgal expressing plasmid, was grown, labeled with [^{35}S]methionine for 3 min and disintigrated as indicated in Section 2. Immunoprecipitation, SDS-PAGE and fluorography were done as outlined in Section 2. All steps were done as in experiments in Fig. 3. Lane 1, no addition; lane 2, addition of 250 μg purified β-galactosidase prior to immunoprecipitation.

Table I

β-Galactosidase activity of βgal test proteins in wild-type (BR1) and mutants (BR2, BR3, BR4) of yeast defective in the chymotryptic activity of proteinase yscE

βGal test protein	Specific activity of βgal test protein (U/mg) in strain						
	BR1 (wild type)	BR2 (*pre1-1*)		BR3 (*pre2-2*)		BR4 (*pre1-1 pre2-2*)	
			Ratio		Ratio		Ratio
Arg-βgal	2.3	20.8	9	27.3	11.9	31.1	13.6
Leu-βgal	4	21	5.3	19.2	4.8	35.6	8.9
Tyr-βgal	9.6	36.1	3.8	51.2	5.3	60	6.3
Ub-Pro-βgal	26	78	3	80.3	3.1	91.7	3.5
Ser-βgal	67	91	1.4	107	1.6	100	1.5
Ala-βgal	123	96	0.8	93.2	0.8	70	0.6

Cell extracts were prepared as outlined in Section 2. β-Galactosidase activity was measured with *o*-nitrophenyl-β-D-galactoside as outlined [19]. 'Ratio' designates the ratio of the specific activities of the respective X-βgal protein measured in mutant extract and wild type extract.

mutants (Table I). Also here no significant alterations in activity were found for the metabolically rather stable X-βgal fusion proteins carrying Ser or Ala at the amino-terminus (Table I). The apparently lower activity level of Ala-βgal in the *pre* mutant cells tested cannot be explained at present. It may reflect a different plasmid copy number or expression of the plasmid-encoded protein in wild-type and mutant cells under the conditions tested. Alternatively degradation of this protein by some other proteolytic system may be enhanced in the mutant cells deficient in the chymotrypsin-like activity of the proteasome. The increased steady-state activity levels measured of the metabolically unstable X-βgal proteins in proteinase yscE mutant cells as compared to wild-type is reflected by their protein levels separated on SDS-PAGE and detected by immunoblotting (Fig. 1, see mark). While for instance very little β-gal protein of the highly unstable Arg-βgal, Leu-βgal, Tyr-βgal or Ub-Pro-βgal can be detected immunologically in wild-type cells (Fig. 1A,B,C lanes 1,3,5,7), its content is considerably increased in all proteinase yscE mutant cells (Fig. 1A,B,C lanes 2,4,6,8). Here it reaches nearly the amount of βgal protein found for the most stable constructs in wild-type cells, Ser-βgal and Ala-βgal (Fig. 1A,B,C lanes 9 and 11). These results indicated that the metabolically unstable test-proteins Arg-βgal, Leu-βgal, Tyr-βgal and Ub-Pro-βgal (Fig. 1A,B,C lanes 1–8) were stabilized in proteinase yscE mutants defective in the chymotryptic activity of the enzyme.

To gain final proof of the proteolytic stabilization of the metabolically unstable X-βgal proteins we determined their degradation rates by conducting pulse–chase experiments. Wild-type and mutant cells were briefly labeled with [^{35}S]methionine, and cell lysates were subjected to immunoprecipitation using antibodies specific for βgal. Labeled and precipitated protein was separated by SDS-PAGE. Purified β-galactosidase specifically competed with the labeled uppermost protein band of Ala-βgal expressing cells, indicating this uppermost protein to be β-galactosidase antigenic material (Fig. 2, compare lanes 1 and 2). Comparison of cells which do or do not express plasmid-encoded β-galactosidase shows that the uppermost protein band is indeed β-galactosidase protein (not shown). We thus compared the fate of this antigenic protein in proteinase yscE wild-type strain BR1 and mutant cells (strains BR3 (*pre2-2*) and BR4 (*pre1-1 pre2-2*)) (Fig. 3). As shown previously [15], the metabolically stable test protein Ala-βgal remained stable in wild-type cells during the test period (30 min) (Fig. 3D, lanes 1–3) whereas proteins known to be metabolically unstable in wild-type cells as are Arg-βgal, Leu-βgal and Ub-Pro-βgal [15] disappeared rapidly with time (Fig. 3A, B, C, lanes 1 to 3). In contrast proteinase yscE mutant cells BR3 (*pre2-2*) or BR4 (*pre1-1 pre2-2*) led to complete stabilization of these in wild-type unstable test proteins (Fig. 3A, B, C, lanes 4 to 9).

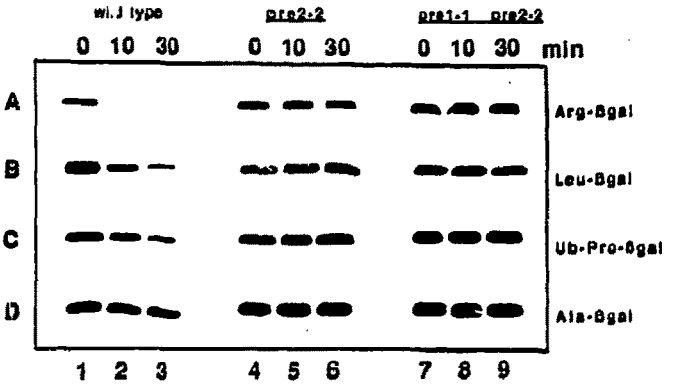

Fig. 3. Degradation of X-βgal test proteins in wild type and mutant cells defective in the chymotryptic activity of proteinase yscE. Cells were grown, labeled with [^{35}S]methionine and disintigrated as indicated in Section 2. Immunoprecipitation, SDS-PAGE and fluorography were done as outlined in Section 2. Labeling was done for 3 min (time point 0 (lanes 1,4 and 7)) and thereafter cells were chased with non-radioactive methionine (10 mM) for 10 min (lanes 2,5 and 8) and 30 min (lanes 3, 6 and 9). Test proteins used were: Arg-βgal (A), Leu-βgal (B), Ub-Pro-βgal (C) and Ala-βgal (D). Degradation of test proteins in wild-type cells (BR1) lanes 1,2,3; degradation in *pre2-2* mutant cells (BR3) lanes 4,5,6; degradation in *pre1-1, pre2-2* double mutant cells (BR4) lanes 7,8,9. Variation in band intensities of wild type and mutant cells at time-point zero are due to different exposure times of gels originating from independent experiments.

4. DISCUSSION

We have shown that mutations in subunits of proteinase yscE conferring chymotryptic activity result in metabolic stabilization of otherwise unstable proteins degraded via the N-end rule pathway. Mutations in the *PRE1* or in the *PRE2* gene show several-fold increased activities (Table I) and protein levels (Fig. 1) of Arg-βgal, Leu-βgal, Tyr-βgal and Ub-Pro-βgal as compared to wild-type. Combination of the two mutations *pre1-1* and *pre2-2* leads to an even tighter phenotype. The metabolically unstable βgal proteins are more enriched in activity and protein over wild-type than in the single mutants (Table I, Fig. 1). Degradation rates of the metabolically unstable βgal proteins were directly tested in a single mutant (*pre2-2*) and the double mutant (*pre1-1 pre2-2*) defective in both subunits necessary for chymotryptic activity of the enzyme. Clearly, degradation of the in wild-type highly unstable Arg-βgal, Leu-βgal and Ub-Pro-βgal proteins is dramatically reduced in the mutant strains. They appear to be stable during the time tested (Fig. 3A,B,C lanes 4–9). We tested the stability of metabolically unstable βgal proteins also in strain BR2 carrying the *pre1-1* point mutation. As expected, the engineered proteins were completely stabilized (not shown). The Arg-βgal-, Leu-βgal- and Tyr-βgal proteins are ubiquitinated prior to degradation via the *UBC2* encoded ubiquitin-conjugating enzyme [25,26] whereas Ub-Pro-βgal is further ubiquitinated via the *UBC4-UBC5* encoded ubiquitin-conjugating enzymes [18] prior to degradation. Thus, our experiments

strongly suggest that proteinase yscE is involved in degradation of ubiquitinated proteins derived from two different ubiquitin-mediated proteolytic pathways in vivo.

A central regulatory protein known to be ubiquitinated is cyclin, a protein which undergoes programmed rapid degradation for progression of the cell cycle to occur [28]. The capacity of proteinase yscE to mediate degradation of ubiquitinated proteins derived from different pathways may suggest that the proteasome represents the final target for degradation of ubiquitinated proteins in general and thus also of ubiquitinated cyclin. Indeed, the lethality of null mutations in different subunits of proteinase yscE [8,24,29–31] of cells would support such a central function for the proteasome.

A general involvement of proteinase yscE in ubiquitin-mediated proteolysis would also predict the short-lived Matα2 transcriptional regulator of mating type in yeast cells, which undergoes ubiquitination [32], to be a substrate of proteinase yscE.

Even though the degradation of the engineered test proteins was blocked in the proteinase yscE mutants defective in the chymotryptic activity, the proteins accumulating did not show the pattern of multiply ubiquitinated proteins. This observation is unexpected as the bulk of proteins accumulating in proteinase yscE mutants with chymotryptic deficiency under stress conditions are ubiquitinated [8,24]. An explanation for this phenomenon might be that proteinase yscE has some function in the ubiquitin conjugation reaction of N-end rule substrates, which is defective in the mutants tested. Alternatively, N-end rule substrates which are not hydrolyzed by proteinase yscE are subject to rapid removal of the ubiquitin moieties by the ubiquitin C-terminal hydrolases. Also a limited cleavage step by one of the remaining activities in the mutant proteasome (the trypsin-like or peptidyl glutamyl-peptide hydrolizing activity) removing a short amino-terminal peptide chain containing the ubiquitin moiety from the test proteins cannot be excluded.

It is a challenging task for our future research to uncover the different proteolytic pathways the proteasome is involved in, and to correlate the different structural components of the enzyme complex – some of which are under heavy debate [33–35] – with their function in the degradation reactions.

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