THE PROTEASOME AND PROTEIN DEGRADATION IN YEAST

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20S PROTEASOMES: ACTIVITIES, GENES AND PROTEINS

In 1984 a high molecular mass multisubunit protease complex was isolated from *Saccharomyces cerevisiae* [Achstetter *et al.* 1984] which proved to be the yeast homologue of the 20S proteasome complexes found in all eukaryotic cells [Kleinschmidt *et al.* 1988]. The yeast 20S proteasome is able to cleave chromo- and fluorogenic peptides at the carboxyterminus of hydrophobic, basic or acidic amino acids (chymotrypsin-like-, trypsin-like- and peptidyl-glutamyl-peptide hydrolyzing activity, respectively) [Heinemeyer *et al.* 1991]. The yeast 20S proteasome is composed of different subunits, showing a set of protein bands in the SDS-PAGE with molecular masses ranging from 20 to 35 kDa. They can be separated into 14 protein spots after two-dimensional gel electrophoresis [Heinemeyer *et al.* 1991]. Genes named Y7, Y13, PRS1 and PRS2 (independendly cloned as Y8 and SCL1) were cloned and sequenced on the basis of protein sequences of 20S proteasome subunits, genes named PRS3, PUP1, PUP2 and PUP3 were sequenced by chance [for summary see Hilt *et al.* 1993b]. We cloned the β–type genes PRE1, PRE2, PRE3 and PRE4 by complementation of mutants defective in the chymotrypsin-like- (pre1 and pre2 mutants) or the PGPH-activity (pre3 and pre4 mutants) of the proteasome [Heinemeyer *et al.* 1991, Heinemeyer *et al.* 1993, Hilt *et al.* 1993a, Enenkel *et al.* 1994]. Additionally we cloned two α–type genes PRE5 and PRE6 using peptide sequences derived from purified proteasome subunits, extending the number of yeast 20S proteasome subunit genes to 14 [Heinemeyer *et al.* 1994]. The now known 14 proteasomal subunits show structural relationships to each other and to those of other species but are not similar to any other known protein. According to their degree of sequence similarity and their relationship to either the α– or the β–subunit of the *Thermoplasma* proteasome they can be grouped

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into seven α–type and seven β–type proteins. We propose that the known 14 yeast proteasomal subunits most likely represent the complete set of proteins constituting the 20S core complex in this organism [Heinemeyer *et al.* 1994]: (i) 14 cloned yeast proteasomal genes nicely correspond to the 14 protein spots obtained after two-dimensional separation of the subunits of the purified yeast 20S proteasome [Heinemeyer *et al.* 1991]. (ii) The seven α–type and seven β–type subunits found in yeast can easily be arranged into a structure with a two-fold a7b7 symmetry as found in the ancestral archaebacterial proteasome [Puhler *et al.* 1992]. (iii) Multiple alignment of all known eukaryotic 20S-proteasome sequences yields a dendrogram which clearly shows 14 subgroups (seven α–type- and seven β–type), each of the subgroups containing a single yeast member. According to the structure proposed for proteasomes of higher eukaryotes [Kopp *et al.* 1993, Schauer *et al.* 1993] the yeast 20S proteasome most probably constitutes a complex dimer with C2-symmetry. Within such a structure each of the 14 different subunits is used two times and occupies two defined positions. The calculated molecular masses of the 14 yeast proteasome subunits ranging from 21.2 to 31.6 kDa are in agreement with molecular masses of the protein bands found in SDS-PAGE studies of purified yeast 20S proteasomes [Tanaka *et al.* 1989, Kleinschmidt *et al.* 1988]. Proteasomes most probably represent a completely new type of proteolytic enzymes. Genetic studies in yeast link certain β–type subunits to different proteolytic activities. Intact Pre1 and Pre2 proteins are necessary for the chymotrypsin-like activity [Heinemeyer *et al.* 1991, Heinemeyer *et al.* 1993] whereas intact Pre3 and Pre4 proteins are necessary for the PGPH activity [Enenkel *et al.* 1994, Hilt *et al.* 1993a]. These results support the idea, that proteolytically active sites may be formed by interaction of two side by side arranged subunits. On the other hand active sites may be located at individual subunits within the proteasome. In this case inactivation may also be caused by conformational changes induced by a mutation in a protein located next to the catalytic subunit. So far only β–type subunits have been found to contain mutations which lead to defects of the 20S proteasome's peptide cleaving activities. By analogy to the archaebacterial "urproteasome" with its β–subunits forming the inner two rings of the cylindrical particle [Puhler *et al.* 1992], the sites of proteolysis in eukaryotic proteasomes are most probably located in the central rings made up of the β–type subunits. It is tempting to speculate that degradation of the finally unfolded proteins occurs in the hole of the 20S cylinder particle [Hilt & Wolf 1992].

THE 26S PROTEASOME

In 1986 a 26S protease complex which degrades ubiquitinylated proteins *in vitro* had been purified from rabbit reticulocytes [Hough *et al.* 1986]. Studies in several higher eukaryotes demonstrated that this 26S protease complex consists of the 20S proteasome as a core and additional subunits attached at both ends of the 20S cylinder [Peters *et al.* 1993]. Strong evidence appeared that this larger proteinase complex (now called 26S proteasome) also exists in yeast: (i) Indication for the function of a 26S proteasome in the yeast S. *cerevisae* is provided by the fact that mutants with defects in 20S proteasome typic peptide cleaving activities are also defective in the degradation of ubiquitinylated proteins in vivo, which are specific *in vitro* substrates of the 26S proteasome [Heinemeyer *et al.* 1993, Heinemeyer *et al.* 1991, Hilt *et al.* 1993a, Richter-Ruoff *et al.* 1992, Seufert & Jentsch1992]. (ii) In recent work a 26S protease complex has been purified from yeast, which exhibits peptide cleaving activities of the 20S proteasome but is in addition able to degrade ubiquitinylated proteins *in vitro* [Fischer *et al.* 1994].

PROTEASOME FUNCTIONS

Proteasomes Are Essential to Life

Proteasomes are essential tools of the yeast cell. In 13 cases chromosomal deletion of one of the 14 different yeast 20S proteasome subunits was lethal. Spores derived from different heterozygous diploids carrying a null mutation of one of several 20S proteasome genes were able to germinate, but in each case cell growth stopped after 2-3 cell divisions [Heinemeyer *et al.* 1994, Hilt *et al.* 1993a]. We suggest that chromosomal deletion of one of the 20S proteasome genes abolishes the assembly of the 20S and 26S proteasome complex and therefore leads to complete loss of all proteasome functions.

Proteasomes Act in Stress-Dependent and Ubiquitin-Mediated Proteolytic Pathways

Yeast mutants have been used to demonstrate the *in vivo* function of the 20S proteasome in stress-dependent and ubiquitin-mediated proteolytic pathways. Mutations affecting the 20S proteasomal genes *PRE1* and *PRE2* lead to defects of the chymotrypsin-like activity [Heinemeyer *et al.* 1991, Heinemeyer *et al.* 1993], whereas mutants of *PRE3* and *PRE4* are defective in the PGPH activtiy of the complex [Hilt *et al.* 1993a, Enenkel *et al.* 1994]. *Pre1-1* single and even more so *pre1-1 pre2-2* as well as *pre1-1 pre4-1* double mutants are sensitive to heat stress [Heinemeyer *et al.* 1991, Heinemeyer *et al.* 1993, Hilt *et al.* 1993a] and also to the arginine analogue canavanine. Under these stress conditions the mutants accumulate ubiquitinylated proteins. It is suggested that heat and canavavine stress lead to the formation of large amounts of abnormal proteins which are ubiquitinylated but remain undegraded in the proteolysis defective 20S proteasome mutants [Heinemeyer *et al.* 1991, Heinemeyer *et al.* 1993, Hilt *et al.* 1993a].

Proteasomes Degrade Short-Lived N-End-Rule Substrates.

The function of proteasomes in the degradation of ubiquitinylated proteins *in vivo* has been confirmed by analyzing the degradation of the short-lived protein substrates of the N-end-rule pathway in 20S proteasome mutants [Richter-Ruoff *et al.* 1992, Seufert &Jentsch 1992]. The short-lived N-end-rule substrates which constitute artificial protein substrates in yeast, and are ubiquitinylated prior to proteolysis, are clearly stabilized in yeast 20S proteasome mutants *pre1-1* and *pre2-2* with defective chymotrypsin-like activity [Richter-Ruoff *et al.* 1992, Seufert & Jentsch 1992]. Stabilization is strongly enhanced in *pre1-1 pre2-2* double mutants [Richter-Ruoff 1992]. 20S proteasome mutants also stabilize the short lived Ub-Pro-ß-gal protein [Richter-Ruoff *et al.* 1992, Seufert & Jentsch 1992] which is ubiquitinylated in a N-end-rule independent pathway [Johnson *et al.* 1992].

IN VIVO SUBSTRATES OF THE PROTEASOME

Unassembled Proteins: Proteasomes Degrade Free α-Subunits of the Fatty Acid Synthase

The yeast fatty acid synthase complex which is composed of 6α- (Fas2) and 6β- (Fas1) subunits, is a rather stable protein ($t_{1/2}$=20h) [Egner *et al.* 1993]. In contrast, in yeast cells containing a chromosomal *fas1* deletion free Fas2 α-subunits are rapidly degraded

($t_{1/2}$=1h). However, in 20S proteasome mutants (*pre1-1*) containing a *fas1* deletion allele the Fas2 protein is clearly stabilized, as compared to *PRE1* wild type cells [Egner *et al.* 1993]. The free α-subunit seems to be recognized as some sort of abnormal protein which is rapidly degraded via the proteasomal pathway.

Proteasomes Degrade Fructose-1,6-Bisphosphatase

Proteasome dependent degradation of a metabolic enzyme has been shown in the case of fructose-1,6-bisphosphatase which is a key enzyme in gluconeogenesis. This enzyme is subject to catabolite inactivation: upon addition of glucose to yeast cells, which have been grown on a non-fermentable carbon source, fructose-1,6-bisposphatase is inactivated by phosphorylation and then rapidly degraded ($t_{1/2}$=1 h). In *pre1-1* 20S proteasome mutants, fructose-1,6-bisposphatase is strongly stabilized. In *pre1-1 pre2-1* double mutants defective in two subunits of the 20S proteasome, degradation of fructose-1,6-bisposphatase is nearly absent [Schork *et al.* 1994].

Proteasomes Degrade the Yeast MATα2-Repressor

Proteasomes are also needed for degradation of regulatory proteins: This has been demonstrated in yeast for the MATα2-protein which is required for mating type differentiation of this organism. The MATα2-protein is responsible for repression of MATa specific genes in haploid MATα cells and of haploid specific genes in diploid cells. The very short-lived MATα2-repressor ($t_{1/2}$=5min at 30^{0}C) [Hochstrasser & Varshavsky 1990] contains two independent destruction boxes and is ubiquitinylated via the ubiquitin conjugating enzymes Ubc4/Ubc5 and Ubc6/Ubc7 [Chen *et al.* 1993, Hochstrasser &Varshavsky 1990]. The function of proteasomes in degradation of the MATα2-repressor has been demonstrated by the stabilization of this protein in 20S proteasome mutants (*pre1-1* and *pre1-1 pre2-2*) [Richter-Ruoff *et al.* 1994].

PROTEASOMES FUNCTION IN YEAST CELL CYCLE CONTROL

Multiple kinase complexes formed by the association of different types of cyclins with a single kinase subunit (Cdc28) are crucial for cell cycle control in yeast [Nasmyth 1993]. The appearance and disappearance of particular kinase forms is regulated by the synthesis and proteolytic degradation of specific cyclins during different phases of the cell cycle. Certain β–type cyclins (Clb1-4) are important for function and assembly of the spindle apparatus during mitosis. Such β–type cyclins are thought to be degraded by proteasomes via ubiquitin dependent pathways: Studies of rapid mitotic degradation of β–type cyclins revealed an aminoterminally located highly conserved stretch of nine amino acids (designated as the "destruction box ") and ubiquitin modification prior to proteolysis [Glotzer *et al.* 1991]. We now uncovered a link between proteasome and Clb2 function. Yeast cells with a proteolytically stable form of the cyclin Clb2 are arrested in the mitotic telophase [Nasmyth 1993, Surana *et al.* 1993]. Overexpression of a single *CLB2* copy from the *GAL1* promoter is tolerated by yeast cells while overexpression of four copies of *CLB2* is lethal [Surana *et al.* 1993]. However in proteolysis defective 20S proteasome *(pre1-1)* mutants, overexpression of even one copy of *CLB2* leads to a halt in cellular growth [Richter-Ruoff & Wolf 1993] suggesting that proteasome dependent proteolysis is needed for control of the cell cycle.

CONCLUDING REMARKS

Thus using yeast as a model organism of the eukaryotic cell, the necessity of the proteasome for life and its involvement in the ubiquitin pathway of protein degradation and in central cellular functions *in vivo* could be shown for the first time. It can be expected that the action of the proteasome as the counterpart of the ribosome will be found in many more essential cellular processes.

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