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Understanding Proteasome Assembly and Regulation: Importance to Cardiovascular Medicine

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Abstract

The cardiac proteasome is increasingly recognized as a complex, heterogeneous and dynamic organelle contributing to the modulation of cardiac function in health and diseases. The emerging picture of the proteasome system reveals a highly regulated and organized molecular machine integrated into multiple biological processes of the cell. Full appreciation of its cardiovascular relevance requires an understanding of its proteolytic function as well as its underlying regulatory mechanisms; of which assembly, stoichiometry, post-translational modification and the role of the associating partners are increasingly poignant.

Keywords

protein degradation; complex assembly; proteolytic activity

Introduction

The mammalian protein degradation machinery is dominated by the proteasome, as it endoproteolytically cleaves over 70% of intracellular proteins (Rock *et al.* 1994). The core of this multimeric protease is a duplex of two sets of fourteen subunits, housing duplicate sites of trypsin-like, caspase-like and chymotrypsin-like peptidase activities. Termed the 20S proteasome, its gated pores maintain the complex in a latently active state, permitting only limited proteolysis, possibly through the exposed hydrophobic residues of oxidized and denatured proteins (Widmer *et al.* 2007). Additional protein complexes such as the 19S and P131 bind to the 20S proteasomes to respectively activate or inhibit the complex (Zais *et al.* 2002), while the binding of PA200, PA28 and PR39 changes the proteolytic cleavage patterns (Kloetzel 2004, Cascio and Goldberg 2005, Gaczynska and Osmulski 2005). As work turns towards the proteasomes' complexity and regulation, it is increasingly apparent that they are engaged in a fundamental and specialized role in the cardiovascular system. Of particular interest are the perturbations in proteasome activities associated with cardiovascular disease phenotypes. Despite several controversial reports, there is an emerging consensus that injured myocardium (e.g., myocardial ischemia reperfusion injury, left ventricular dysfunction) is concomitant with an attenuated proteolytic function in the heart (Bulteau *et al*. 2001, Gurusamy

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et al. 2007, Voortman and Giaccone 2006, Luss *et al.* 2002, Pye *et al.* 2003, Stansfield *et al.* 2007).

Functional Proteasomes Require a Highly Regulated Assembly

The assembly process begins with single subunits. Expression of the proteasome subunits is a coordinated and controlled process orchestrated through a number of signaling mechanisms and cellular sensors. Under physiological conditions, the Rpn4 subunit of the 26S proteasomes binds to the proteasome-associated control element (PACE) through a zinc finger motif, activating expression of α, β and 19S subunits (Mannhaupt and Feldmann 2007). With a half-life of 2 min, Rpn4 is degraded by the proteasomes and enables the subunit to dynamically stabilize proteasome levels within the cell (Ju *et al.* 2001). Proteasome transcription is also governed by the proteolysis-inducing factor (PIF), a sulphated glycoprotein excreted by tumors that regulates muscle mass (Russell *et al.* 2007). In murine myotubes, PIF induces proteasome expression through 14(*S*)-hydroxyeicosatetraenoic acid and the subsequent PKC_{α} mediated activation of nuclear factor-κB DNA-binding activity (Wyke *et al.* 2004). By modulating proteasome expression, the cell produces a pool of free subunits from which proteasome assembly can rapidly respond to dynamic regulatory input.

Mature proteasomes require a highly regulated assembly process that must compliment the tissue type and its multitude of functions (Figure 1). Some associating proteins serve as the molecular scaffolds and chaperones that regulate the assembly of the 20S and 26S proteasomes, providing another mechanism for controlling cellular proteasome levels (Schmidt *et al.* 2005). The proteasome assembly chaperones 1 and 2 (PAC1 and PAC2) dimerize, and then bind α5 and α7 to initiate the formation of the α ring (Hirano *et al.* 2006). Subsequent arrangement of the α ring and the initial β subunits is aided by a dimer of proteasome assembly chaperones 3 and 4 (PAC3 and PAC4) as they bind α2, β3 and β4 (Le Tallec *et al.* 2007). At this point, incorporation of either constitutive or inducible β subunits may take place, thus altering the proteolytic characteristics of the proteasomes (Fuchs *et al.* 2007). A protein that associates with proteasomes in yeast (Ump1p) and humans (hUmp1p, proteassemblin, POMP) is believed to assist with the formation of the remaining β subunits to form the half proteasome (Jayarapu and Griffin 2004). Maturation of the complex through the transitional 13S and 16S intermediates is assisted by hUmp1p and stabilized by the heat shock protein HSC73 (Schmidtke *et al.* 1997). Proteolytic processing of the constitutive or inducible β propeptides follows the junction of two half-proteasomes forming the proteolytically active 20S proteasome (Jayarapu and Griffin 2004).

Binding of the 19S activator protein complex to either end of the 20S forms the 26S and 30S proteasomes, a process that is facilitated by the Pno1p and Nob1p chaperones (Tone and Toh-E 2002). Metabolic control of the 19S is believed to dynamically regulate the 26S proteasomes. Phosphorylation of the ATPase subunits, ser^{120} of RPT6 in particular, by PKA correlates with increased chymotryptic and tryptic activity, and is reversible by treatment with serine/threonine phosphatase PP1γ. Alternatively, *O*-GlcNAcylation of Rpt2 can act as a master switch, shutting off proteolytic activity upstream of proteasome phosphorylation (Zhang *et al.* 2007). Targeting of poly-ubiquitinated proteins through additional protein complexes further integrates the proteasomes' activities with the varying cellular machinery of the heart and different tissues (Dong 2004 *et al.*, Verma *et al.* 2004). Although the proteasome assembly process is beginning to be unraveled, the impact it has on proteasome activity or its regulatory relevance remains to be determined.

Stoichiometry and Quantification of the Proteasome Complexes

Elucidating the relative amounts of all the components of the ubiquitin proteasome system is necessary for mapping its regulation. Establishing the ratios of *any* of these proteins as they

pertain to the proteasomes has yet to be fully accomplished, but the attempts to date provide for an excellent groundwork. Much of the information regarding the 20S stoichiometry and quantification has come from the more traditional biochemical and gel based approaches. Two – dimensional gels of immuoprecipitated 20S proteasomes from Hela cells, utilized incorporated ^{3}H-leucine to identify all 14 constitutive subunits and approximate, via the counts per minute, that all subunits appear in equal stoichiometry (Hendil *et al.* 1993). The subunit ratios of the 26S proteasomes have been documented to change in the gastrocnemius muscle of mice as they age. Expression levels from Northern blots suggests that the relative amount of the α2, α3, β6 and Rpt2 subunit transcripts increases over the course of 34 months, with α2 increasing over 900% (Bardag-Gorce *et al.* 1999). It was also noted by Western blot that the absolute amount of α1 increased by 40% at 29 months before returning back to normal levels by 34 months.

Utilizing a combination of LC-ESI/MS, LC-MALDI/MS, two-dimensional electrophoresis and the Isotope Labeled Affinity Tags (ICAT), levels of rat liver 20S proteasome subunits were compared to those in erythrocyte and U937 cells (Schmidt *et al.* 2005). The ratios of 20S proteasome subunits by ESI/MS in the rat liver gave values with little significant difference, and no values for β2, β5 or the inducible subunits. The MALDI/MS values gave similar ratios with no values for α2, α5, β2 β5, β7 or the inducible subunits, suggesting that most of the subunits are present in equal amounts. Burlet-Schiltz's group has also used a quantitative mass spectrometry approach in conjunction with two-dimensional electrophoresis to investigate 20S proteasome heterogeneity between human erythrocytes and U937 cells (Froment *et al.* 2005). Their use of cleavable ICAT (cICAT) labeled peptides of purified erythrocyte 20S proteasomes produced values ~1% off the expected 0.2 ^{13}C/^{12}C and 20% off the 1.0 ^{13}C/^{12}C; however, the values were consistent between the different ratios, suggesting a 1:1 stoichiometry of the subunits (β7, β1i, β2i, β5i were never detected). Comparison of 20S proteasomes between erythrocyte and U937 cells, α1, α3, α4, α5, α7 and β6 all showed the expected 1:1 ratio, while α2, β1 and β4 in U937 cells were only 15–20% as abundant. Although the subunit ratios are now well established for the proteasome, the lack of quantitative data between tissues and organisms will, we hope, lessen with more accurate and reliable methods of quantification.

Extending beyond the proteasome, a considerable amount of work has focused on quantifying some of the ancillary proteins that interact and potentially regulate the proteasomes. Lan Huang's group quantified the 26S proteasomes and its associating partners in arginine auxotrophic yeast strains using stable isotope labeling with amino acids (SILAC) (Guerrero *et al.* 2006). Heavy and light 26S proteasomes were purified with the use of tandem affinity columns, mixed, digested in solution and subjected to LC-MS/MS analysis. The relative abundance ratios of the peptides (L/H) were calculated by either monoisotopic peak intensity or area. Of the proteasome subunits, the 19S Rpt6 showed a ratio of 5.1 and the 20S α1 was 3.2, a difference suggesting a difference in growth between wild type and auxotrophic strains. A second effort from their line of work focused on quantifying the interacting proteins of the proteasomes. By differentiating between proteins that were pulled down with the 26S proteasomes and those that interacted with the complex after purification, their group identified 35 stable interacting proteins and 16 dynamic interacting proteins. Establishing the abundance and stoichiometry of the associating partners will enable the sorting of intersecting and regulatory pathways of the proteasomes in any tissue.

Current Understanding of the Mammalian Cardiac Proteasomes

A model of the cardiac proteasomes is emerging with the complexity and organization expected from such an integral molecular machine (Figure 2). A novel approach separating 20S proteasomes using in-solution isolectric focusing in a laminar flow indicates that the heart maintains a wide variety of proteasomes, differing in molecular composition and proteolytic

activity from those in the liver. A majority of these cardiac proteasomes have an isolectric point of 5.26, compared to a pI of 5.05 for most of the proteasomes, and are largely reflective of their phosphorylation complement. The 20S proteasome subpopulations within each tissue also demonstrated unique proteolytic activity profiles and inducible subunit compositions (Drews *et al.* 2007). Proteasomes exhibiting distinctly varying hydrophobic and basic protease activities with different targets during oxidative stress injury of the rat heart may also suggest specific proteasome functions associated with individual subpopulations (Gurusamy *et al.* 2007). Evidence suggesting that proteasomes exist as a diverse and distinct range of forms within the cardiomyocyte is supported by functional proteomic studies of the 26S proteasomes. All inducible subunits were profiled in purified 26S cardiac proteasomes as well as alternate splicing of Rpn10, N-terminal acetylation of Rpn1, Rpn 5, Rpn 6, Rpt 3, Rpt 6, α2, α5, α7, β3 and β4, myristolation of Rpt 2 and phosphorylation of α7 (Gomes *et al.* 2006). Another element of proteasome heterogeneity in the heart and intimately related to the post-translational state of the proteasome is the involvement of associating partners. Of the many proteins that have been found to stably interact with the proteasome, PKA and PP2A have been found to respectively increase and decrease the three proteolytic activities of the cardiac 20S in a substrate-specific fashion (Zong *et al.* 2006). As the capacity of the cardiac proteasomes emerges, studying the varying array of proteasomes within the cell and between tissue types will be critical to understanding protein quality control in mammalian systems. Maintaining a wide variety of proteasome subpopulations affords a cell the simultaneous versatility and regulation that contributes to all tissue types.

The significance of the proteasomes in the cardiovascular system and their relevance to disease is just now beginning to be revealed. Myocardial ischemia reperfusion injury and the corresponding oxidative modification of the proteasome have both been shown to alter the proteolytic activity (Post *et al.* 2006, Bulteau *et al.* 2001). The relationship between oxidized or ubiquitinated proteins and the proteasome directly correlate with postischemic recovery (Powell *et al.* 2005), while some cardioprotective proteins are stimulated during proteasome inhibition (Stangl *et al.* 2002, Townsend *et al.* 2004). Proteasome inhibitors during I/R injury have also been found to decrease myocardial infarct size, reduce leukocyte accumulation and almost entirely eliminate coronary contractile dysfunction (Pye *et al.* 2003, Campbell *et al.* 1999). Collectively, multiple lines of evidence documented a relationship of altered proteasome function in diseased myocardium. However, it remains controversial whether reduced proteasome function is beneficial or detrimental (Luss *et al.* 2002, Voortman and Giaccone 2006, Stansfield *et al.* 2007). In our view, such inconsistencies arise from the deficit in our current knowledge of the proteasome complexes, specifically with respect to their molecular structure and underlying regulatory mechanisms. This paucity of information makes elucidating its complete role in the cardiovascular system imperative for advancements in cardiac biology and medicine.

Concluding Remarks

In summary, alterations in proteasome assembly or activity may be achieved by manipulating the ratios or availability of assembled subunits. The emerging realization of the proteasomes' significance to cardiovascular health underscores the importance of a careful examination of this organelle. A preliminary model of cardiac proteasome complexes suggests multiple regulatory facets, each of which remains to be fully elucidated. Every molecule involved in assembling the proteasome, or altering its activities and functions, provides the complex with additional levels of regulation and increases its functional capacity within the cell. With the addition of post-translational modifications and cellular localization, the proteasome achieves a functional diversity crucial to cellular activities. Gaining insight into the precise stoichiometry and quantities of the complex in each tissue will aid in the elucidation of its regulation and response to pathology. Advances in mass spectrometry and the corresponding

methods in protein quantification will provide great insight into the regulation of the proteasome and its binding partners.

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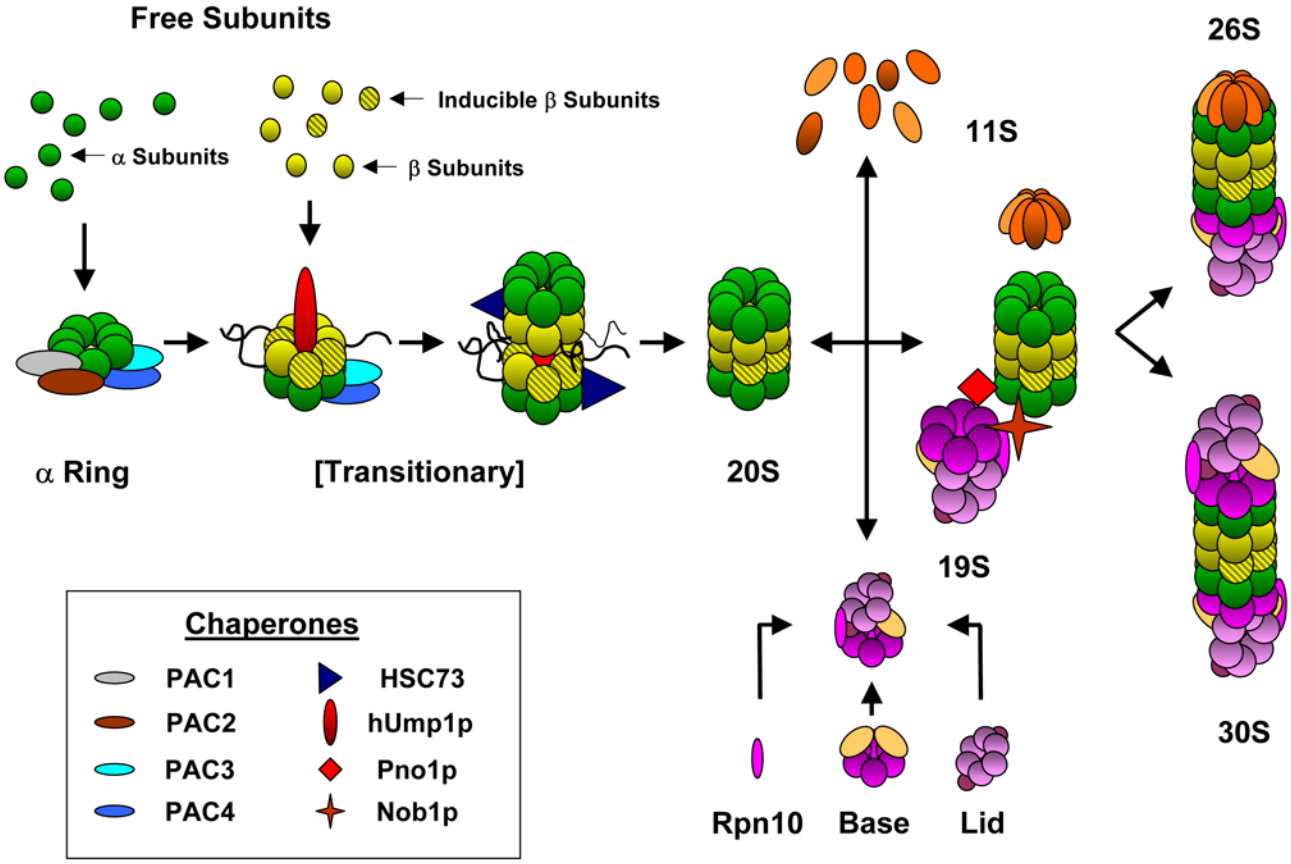

Figure 1. Flowchart of proteasome assembly

Proteasome assembly is mediated by a growing list of molecular chaperones. The currently accepted view of proteasome assembly begins with the recruitment of free α subunits into an α ring by the pac1/pac2 heterodimer (Hirano *et al.* 2006); subsequently, completion of the α ring and addition of the constitutive or inducible β subunits to form a transitionary half-proteasome is aided by the pac3/pac4 dimer (Le Tallec *et al.* 2007) In addition, Hsc73 and hUmp1p aid in the junction of two half-proteasome complexes and the proteolytic processing of the propeptides on some of β subunits yield a latently proteolytically active 20S (Jayarapu and Griffin 2004, Schmidtke *et al.* 1997). Finally, the regulatory complexes, namely the 11S, 19S, or PA200, may be mated to either end of the 20S proteasome to form functional complexes (26S or 30S). For example, the 19S complex is formed from a base of ATPase subunits, Rpn10 and a lid of non-ATPase subunits. Combination of the 19S with the 20S proteasome is facilitated by the Pno10 and Nob1p chaperones and can interface once to form the 26S or twice to form the 30S proteasomes (Tone and Toh-E 2002). The 11S heptameric complex is composed of α, β, and γ subunits and can also interface with the 20S proteasome, forming hybrid complexes of alternative function (Kloetzel 2004). Inset: chaperones that have been putatively identified to aid in proteasome assembly.

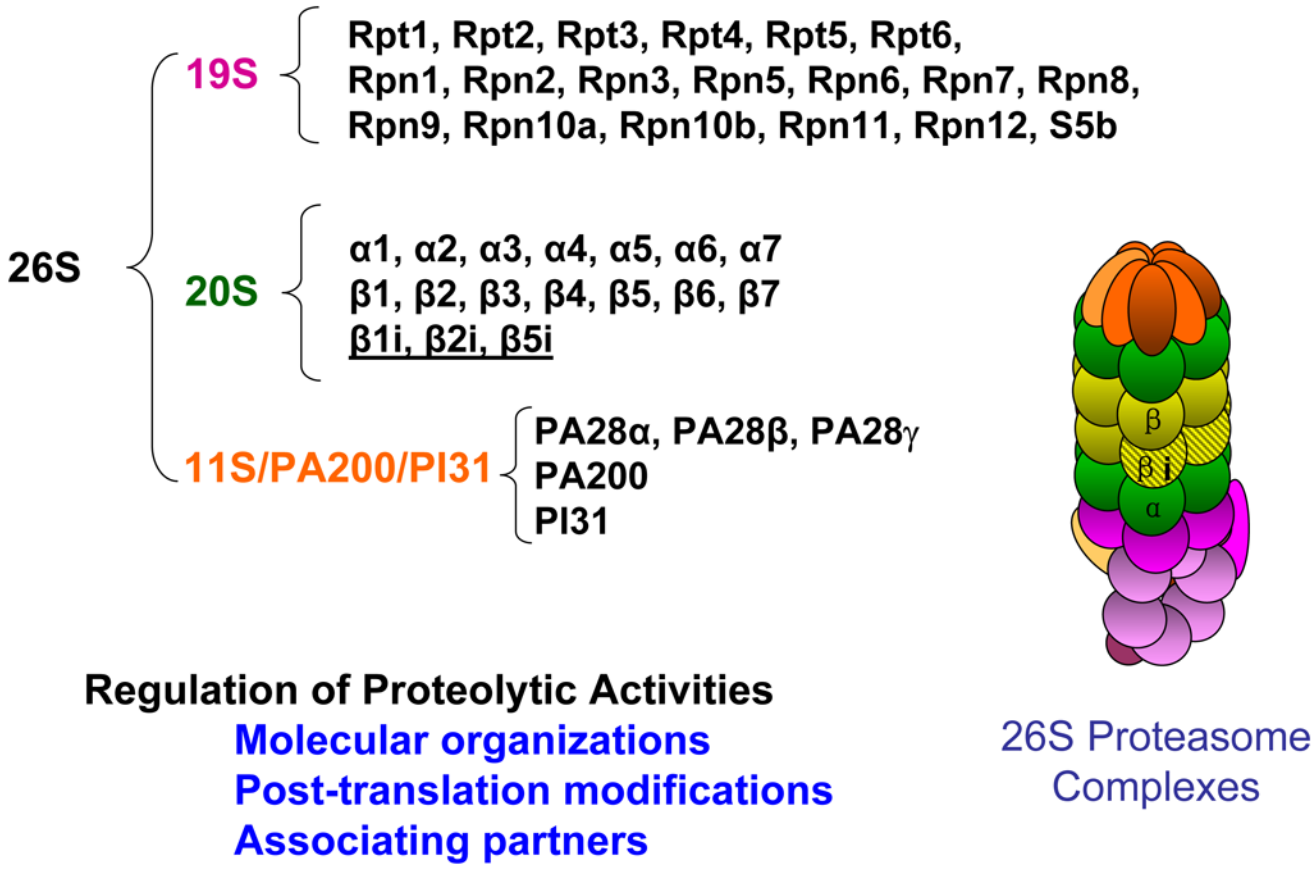

Figure 2. The model of cardiac proteasomes

Cardiac proteasome complexes were purified from the murine heart by multidimensional chromatography and analyzed by mass spectrometry to yield a profile of components. The mammalian heart displays a heterogeneous mix of proteasomes, as all14 constitutive subunits (α1, α2, α3, α4, α5, α6, α7, β1, β2, β3, β4, β5, β6, β7) and all three of the inducible subunits (β1i, β2i, β5i) were found in the 20S proteasomes. Of the 19S proteasome complex, a total of 19 subunits have been identified. The six ATPase subunits include Rpt1, Rpt2, Rpt 3, Rpt4, Rpt5 and Rpt 6. The remaining 19S subunits are all non-ATPases and include Rpn1, Rpn2, Rpn3, Rpn4, Rpn5, Rpn6, Rpn7, Rpn8, Rpn9, Rpn10a and its isoform Rpn10b, Rpn11, Rpn12 and S5b. In addition to the 19S activator, the three subunits of the 11S activator (PA28α, PA28β, PA28γ), PI31 inhibitor and the PA200 regulatory complexes were also found associated with the purified proteasomes. These proteomic analyses of proteasome complexes helped us understand the mechanistic insights of this protein degradation machinery. Our investigations demonstrate that cardiac proteolytic function is regulated via at least three mechanisms, i.e., molecular organization, post-translational modification and the associating partners of the cardiac proteasomes (Gomes *et al.* 2006).