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**EXPLORE THE MURINE CARDIAC 20S PROTEASOMES:
MOLECULAR COMPOSITION AND REGULATION**

By

Chenggong Zong
B.S., Ocean University of China, 2000

A Thesis
Submitted to the Faculty of the
Graduate School of the University of Louisville
in Partial Fulfillment of the Requirements
For the Degree of

Doctor of Philosophy

Department of Physiology and Biophysics
University of Louisville
Louisville, Kentucky

December, 2005

**Explore the Murine Cardiac 20S Proteasomes:
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A Dissertation Approved on

November 9, 2005
Date

By the following Dissertation Committee:

Dissertation Director

DEDICATION

This thesis is dedicated to my parents

Mr. Qingang Zong

and

Mrs. Jiamei Xu

Who have giving invaluable support and educational opportunities.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to my mentor, Dr. Peipei Ping for her extraordinary guidance and consistence support and being such a great role model. Her dedication, brilliance have been such an inspiration to me.

I am also very grateful for having a great graduate advisory committee. Dr. Irving Joshua, Dr. William Wead, Dr. Stanley D'Souza and Dr. Gregg Rokosh have provided excellent advice and support throughout my training process. Many thanks to the rest of faculty and administration staff in Department of Physiology and Biophysics, who have been so helpful.

My special appreciate to my colleague in Dr. Ping's group for their help and friendship: Dr. Jun Zhang, Dr. Thomas Vondriska, Dr. Chris Baines, Dr. Xinan Cao, Dr. Guang-wu Wang, Mr. Ernest Cardwell, Dr. Xin Qiao, Dr. Ming Lu, Dr. Aldrin Gomes, Dr. Xiaohai Li, Mr. Glen Young, Dr. Oliver Drews, Dr. David Liem, Ms. Dawn Pantaleon, Mr. Gabe Bernard.

Finally, I would like to express my appreciation to my parents for unconditional love, support, mentorship and even sacrifice. It has been invaluable for me.

Thank you all!

ABSTRACT

EXPLORE THE MURINE CARDIAC 20S PROTEASOMES: MOLECULAR COMPOSITION AND REGULATION

Chenggong Zong

November, 2005

20S proteasome, essential component of protein degradation mechanism, is important to maintain homeostasis. Its malfunctions have been associated with several pathological conditions. This study presents an extensive study of murine cardiac 20S proteasome. Using biochemical methods, 20S proteasome have been purified to 95%. Proteomic study identified all 20S proteasome subunits. Endogenous phosphorylation was also documented. Furthermore, several associating kinases and phosphatase were identified. They regulated its activities. In PKC ϵ over-expression mice, 20S proteasome expression level was up-regulated, but its peptidase activities did not increase. α B crystallin were recruited to PKC ϵ subproteome in the transgenic mice, which also associated with 20S proteasome. This association was enhanced in the transgenic mice and has been reported to inhibit 20S proteasome activities. It suggested α B crystallin play a role in cardiac 20S proteasome regulation.

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CHAPTER I

GENERAL INTRODUCTION AND OVERVIEW

Literature Review

Proteasomes: An Essential Protein Degradation Machinery

The proteasome is a multi-meric protease complex. In the early days of proteasome study, it was also known as a “multicatalytic proteinase”, “macroxyproteinase” or “alkaline proteinase”. Further investigations proved all those names associate with the same protein complex. This is an essential multimeric complex in cell biology. Its importance is highlighted by the fact that multiple subunits of 20S proteasome are essential for yeast survival by knock down study (Heinemeyer, Trondle et al. 1994). In the cell, the proteasome exists in multiple forms with different sedimentation rates. One form of the proteasome with a sedimentation coefficient of 20S is the minimal unit to have proteolytic activities and is called the 20S proteasome or core particle. The core particle with a 700kDa regulatory protein complex (regulatory particle or PA700) binding on either or both ends has a higher sedimentation coefficient of 26S and is named the 26S proteasome, accordingly. Besides the 26S proteasome, the core particle can associate with other regulatory protein/protein complex, which all contribute to the plasticity of the proteasome. These include the 11S regulatory complex and PA200. The recruitment of different regulatory complexes to the core particle constitutes the variety of proteasome population within the cell, and the choice

of regulatory complexes is an important mechanism to tune the intracellular protein degradation pathway (Coux, Tanaka et al. 1996; Bochtler, Ditzel et al. 1999; Glickman and Ciechanover 2002). In depth understanding of the structure, function and regulation of this essential proteolytic machinery is of great significance.

Mechanisms of Intracellular Protein Turn-over

One of the key features of life is the ability of maintaining homeostasis at the expense of energy. Among the several important aspects of it, protein degradation is an indispensable part. There are two main proteolytic systems, which execute routine protein catabolism in cells: proteasome and lysosome pathways. In addition, there are Ca²⁺ dependent proteases and apoptosis-related proteases, which are involved in specific signaling events (Debigare and Price 2003).

Lysosome degrades membrane proteins and endocytosed proteins. These activities correspond to cell membrane receptor regulation and cellular defense mechanism against exotic proteins (Glickman and Ciechanover 2002). Mono-ubiquitination serves as tag for selective endocytosis. Ubiquitin, as a shared player in both lysosomal proteins degradation and proteasomal protein degradation, links these two protein degradation pathways. Ubiquitin-dependent lysosomal degradation is recently reviewed (Bonifacino and Traub 2003).

Proteasome is responsible for turning over the majority of the cytosolic and nuclear proteins. Proteins which are to be degraded, are tagged with poly-ubiquitin chains (poly-Ub) by the

cooperation of ubiquitin-activating protein (E1), ubiquitin carrier proteins (E2s) and ubiquitin-protein ligases (E3s and E4s) (Hershko and Ciechanover 1998). Poly-ubiquitinated proteins are recognized by poly-Ub receptor in the 26S proteasome (Rpn10) or shuttle proteins (such as Rad23). The poly-Ub is removed, and the tagged-proteins are degraded (Miller and Gordon 2005) into peptides with an average length of 8-9 amino acids. These peptides are either further digested by intracellular peptidases (e.g. Tripeptidyl-peptidase II) into amino acids to be reused; or transported to the ER then represented by MHC I receptor as epitopes, playing important role in immune response (Bochtler, Ditzel et al. 1999).

The Molecular Components and Structure of Proteasomes

Proteasome is in fact a collection of multi-meric proteinases, which are subunits of the 20S core particle. This core particle has been identified in all three branches of organism society: archaeal bacteria, eubacteria and eukaryotes (Hoffman, Pratt et al. 1992; Zwickl, Kleinz et al. 1994; To and Wang 1997; Bochtler, Ditzel et al. 1999; Shibahara, Kawasaki et al. 2002; Wang, Bozdech et al. 2003).

Archaeal bacteria contain an ancient form of proteasome core particle. This particle is made up with two subunits with homology, α and β subunits. They form 4 stack of hapto-rings, with α subunits, which constitute two outer rings and β subunits, which constitute two inner rings (Bochtler, Ditzel et al. 1999). The β subunit is catalytically active, while the α subunit is inactive. Crystallography shows both α and β subunits share the same conformation. Two layers of 5 strands of the anti-parallel β -sheets form the core of both subunits, flanked by 2

anti-parallel α helices on both ends, with an extra α helix on the N-terminus of the α subunits, which compares to the mature form of β subunit (Brannigan, Dodson et al. 1995; Oinonen and Rouvinen 2000). N-terminal pre-peptide of β subunit precursor is auto-proteolytically removed during proteasome assembly, which generates mature β subunit (Zwickl, Kleinz et al. 1994).

Both structural and biochemical studies suggest that proteasome subunits are distinct from well-characterized cysteine proteases, serine proteases, aspartic proteases and metalloproteases (Lowe, Stock et al. 1995; Kisselev and Goldberg 2001). In fact, they belong to a family of proteins called N-terminal nucleophile hydrolases (Ntns). Consistent with the name, N-terminal hydroxyl group of proteins in this family serves as catalytic-active site. The extra N-terminal helix in the α subunit explains its functional impotency (Brannigan, Dodson et al. 1995; Oinonen and Rouvinen 2000). Though catalytically inactive, the α subunit plays key roles in proteasome assembly and substrate specificity. The limited hole (13Å) formed in the middle of heptametrical α ring, only allows certain proteins and peptides to access the proteolytic active sites, which is the structural basis for substrate specificity (Zwickl, Kleinz et al. 1994). Only a few eubacterial contain proteasomes, which are the result of horizontal transfer events (Bochtler, Ditzel et al. 1999).

Evolution diversifies proteasome subunits in eukaryotes. In yeast, there are 7 distinct α subunits and 7 distinct β subunits. The classification as α or β family member is both based on the homology between these subunits and ancestor archael bacteria subunits; and the spatial arrangement of the subunit in 20S proteasome. Two copies of each subunits form a 28-mer 4 layers stack (α 1-7, β 1-7, α 1-7, β 1-7). Crystallography studies uncovered the spatial relationship

among different subunits and proposed an unequivocal nomenclature (Groll, Ditzel et al. 1997; Groll, Koguchi et al. 2001). Both α subunits and β subunits of yeast proteasome have major distinction with archbacterial proteasomes. Among the 7 β subunits, 4 of them have N-terminal truncation concurrent with proteasome maturation, and 3 out of these 4 are proteolytically active. These proteolytic β subunits have distinct preferences towards peptide substrates. The specificity of each subunit has been characterized by mutagenesis and inhibitor analysis. The β 1 subunit prefers to digest after acidic amino acids (caspase-like activity); β 2 subunit prefers to digest after basic amino acids (trypsin-like activity); β 5 prefers to digest after hydrophobic amino acids (chymotrypsin-like activity). Unique fluorescent-tag recombinant peptides were designed to study subunit specific peptidase activities (Leu-Leu-Glu-AMC for β 1 subunit; Leu-Ser-Thr-Arg-AMC for β 2 subunit; Leu-Leu-Val-Tyr-AMC for β 5 subunit) (Arendt and Hochstrasser 1997; Heinemeyer, Fischer et al. 1997; Groll, Koguchi et al. 2001; Kisselev and Goldberg 2001). Prepeptide removals are achieved autoproteolytically or executed by neighboring subunits. Though removed upon maturation, these prepeptides play more important role than that in archaeobacteria. Delete mutation of yeast β 5 presequence leads to cell death (Chen and Hochstrasser 1996). N-terminal sequences of α subunits are also significantly different from the archaeobacteria counterpart. They occlude both ends of 20S proteasome, as a direct result of elongated α subunits in the N-terminal sequence. This leaves yeast 20S proteasome predominantly in a latent form (Groll, Ditzel et al. 1997). There is a dynamic balance between the active form and latent form of 20S proteasome. This explains why the 20S proteasome shows

basal peptidase activities in an un-induced condition. This balance has been revealed by atomic force microscopy (Osmulski and Gaczynska 2002; Furuike, Hirokawa et al. 2003). However, the proteasome can be regulated to favor activated form by physiological activators (e.g. PA700, Blm10) (Adams, Crotchet et al. 1998; Schmidt, Haas et al. 2005) or biochemical activators (e.g. SDS, poly-lysine) (Shibatani and Ward 1995; Coux, Tanaka et al. 1996).

In mammals, the 20S proteasome is highly homologous to that of yeast. The sequences of core particle subunits are conserved. Mammalian 20S proteasome also contains duplex of 7 α and 7 β subunits, organized in 4 stacks of hapto-rings. Both crystal graphic and immuno-electron-microscopy studies show the spatial arrangement of subunits are also conserved among species (Groll, Ditzel et al. 1997; Kopp, Hendil et al. 1997; Dahlmann, Kopp et al. 1999; Unno, Mizushima et al. 2002). Both ends of mammalian 20S proteasome are also occluded, which is consistent with the latency of it. The occlusion can be released by 19S (homolog of PA700 in yeast) and PA200 (homolog of Blm10 yeast). Besides similarities, there are increased complexities in mammalian proteasome to accommodate various functional requirements. Three interferon- γ (IFN- γ) inducible proteolytic-active subunits (β 1i/Lmp2, β 2i/MECL-1, β 5i/Lmp7) are coded and expressed upon stimulation, which is involved in immune response. They would replace their constitutive expressing counterparts (β 1, β 2 and β 5 respectively) under IFN- γ regulation (Groettrup, Ruppert et al. 1995; Gaczynska, Goldberg et al. 1996). There is another regulatory complex inducible by IFN- γ unique to mammalian 20S proteasome, instead of yeast companion. 11S complex is an ATP-independent proteasome

activator involving in immune response (Groettrup, Ruppert et al. 1995; Rechsteiner, Realini et al. 2000). The co-existence of constitutive 20S proteasome subunits and their inducible counterparts in mammals, raise the possibility of multiple "mixed" 20S proteasome forms in mammals. Though existence of these forms has been shown, 20S proteasomes comprising either only constitutive subunits (constitutive 20S) or only inducible subunits (immuno-proteasome) are the dominant forms. Mutational studies show that β subunit prepeptides dictate the preference. Compared to constitutive proteasome, immuno-proteasome increases chymotrypsin-like and trypsin-like activities and decreases caspase-like activity. The peptides, generated by immuno-proteasome, have an average length longer than ones generated by constitutive-proteasome. A higher percentage of peptides have a hydrophobic or basic N-terminal amino acid, too. These peptides are better represented by MHC-1 receptor, which is consistence with an enhanced presentation of epitopes. (Groettrup, Ruppert et al. 1995; Rechsteiner, Realini et al. 2000; Kuckelkorn, Ruppert et al. 2002; De, Jayarapu et al. 2003; Kloetzel and Ossendorp 2004; Forster, Masters et al. 2005).

Ubiquitination and The E1-E4 Cascades

Ubiquitin (Ub) is a well-conserved 76 amino acid protein in eukaryotes which is always synthesized in precursor forms either as a polypeptide containing multiple copies of Ub or co-expressed with a ribosomal protein. De-ubiquitinases (DUBs) activate Ub precursor by truncating it. C-terminal glycine carboxyl-group of Ub can be activated by E1 in an ATP dependent manner. There is one E1 gene in both yeast and mouse. Activated Ub transfers from E1 to E2 and then to lysine ϵ -NH₂ group of protein substrate forming an isopeptide bond

as the result of the interplay between E2 and E3. There are tens of E2s and hundreds E3s encoded in mammalian cells to achieve high specificity of Ub tagging. Besides lysine ϵ -NH₂ of protein substrates, activated Ub can also tag ϵ -NH₂ of Ub (K29, K48, K63) linked to substrates, thereby forming a poly-Ub chain. Poly-Ub chain linked by G76/K48 serves as a signal recognized by 26S proteasome, which targets protein substrates to degradation. The biological context of poly-Ub chain formed by G76/K29 or G76/K63 is less well understood. G76/K63 chain has been reported to involved in DNA repair signaling (Hershko and Ciechanover 1998; Myung, Kim et al. 2001; Weissman 2001; Glickman and Ciechanover 2002). E4s are a special group of ubiquitin ligases that selectively add extra Ub to preformed G76/K48 chain. U-box proteins function as E4 in the cell (Hatakeyama and Nakayama 2003).

As opposed to poly-ubiquitination and sequentially degradation of nuclear and cytosolic proteins, membrane proteins can be mono-ubiquitinated, which serves as a sorting signal for vesicle trafficking process. Some of the vesicles merge with lysosome and membrane proteins are degraded. This vesicle trafficking event has been reviewed recently (Katzmann, Odorizzi et al. 2002; Horak 2003).

The specificity of ubiquitin-tagging procedure is exerted by hundreds of E3s. There are two kinds of E3s: HECT domain containing E3s and Ring-finger domain containing E3s. HECT domain containing E3s have a cysteine in the active site, which accepts activated Ub from cognate E2s and passes it to its substrates. Ring-finger domain containing E3s have zinc-finger domains instead of active site cysteine. They align cognate E2s and protein

substrates and catalyze a direct transfer of Ub between them (Hershko and Ciechanover 1998; Deshaies 1999; Myung, Kim et al. 2001; Pickart 2001; Schwechheimer 2004). Deubiquitination is another key mechanism regulating the specificity of ubiquitin tagging process. As mentioned previous, DUBs are required for maturation of ubiquitin. DUBs associates with 26S proteasome removes poly-Ub chains from protein substrates. This facilitates the translocation of substrate into the core particle and recycles ubiquitin. Non-proteasome-associating DUBs remove non-specific ubiquitination and involves in vesicle trafficking/receptor recycle. This partially explains proteins attached by poly-Ub of less than 4 Ub molecules are poor proteasome substrate. There are 5 families of DUBs reported. They work inversely with E3 ligases to make sure proper substrates specificity (Ferrell, Wilkinson et al. 2000; Amerik and Hochstrasser 2004).

The 20S Proteasomes Are Composed of Multiprotein Complexes

Native gel electrophoresis followed by fluorescent substrate overlay, showed the existence of three species of 20S proteasome. Denaturing SDS-PAGE analysis showed all these species contain all subunits of 20S proteasome, while one form with an extra 30kDa band, the other with an extra 160kDa band. 20S proteasome species containing either 30kDa proteins or 200kDa protein showed enhanced peptidase activity. The 30kDa proteins are highly suggested to be 11S subunit α and β , while 160kDa protein is suggested to be PA200 (Hoffman, Pratt et al. 1992; To and Wang 1997). Purification of these different forms of 20S proteasome from cells proposes that 11S and PA200 regulate 20S proteasome activity *in vivo*.

In vertebrates, existence of IFN- γ inducible subunits increases the complexity of intracellular 20S proteasome population. Immuno-proteasome is induced by IFN- γ and formed via *de novo* synthesis pathway. It has also been identified in other organs, instead of immuno-specific organs, including the heart. Besides playing a key role in immune response, other aspects of regulation evoked by immuno-proteasome induction are not clear (Bose, Brooks et al. 2001; Kloetzel and Ossendorp 2004).

In a 2D electrophoresis (2DE) gel, purified 20S proteasome show more spots than expected according to the number of gene encoding 20S proteasome subunits (14 for yeast, 17 for vertebrate). Mass spectrometry study show several proteasome subunits are each represented by multiple spots on the 2D gel, respectively. This indicates the presence of extensive post-translational modification regulations and the presence of a population of intracellular 20S proteasome with different modifications (Eleuteri, Angeletti et al. 2000; Froment, Uttenweiler-Joseph et al. 2005; Hayter, Doherty et al. 2005).

Regulation of Proteasomal Activities by Post-translational Modification

As mentioned above, selective poly-ubiquitination defined substrate specificity of ubiquitin-proteasome system; while the proteasome is only protein complex that turns over ubiquitin tagged substrates. The 20S proteasome is a stable protein complex that needs a long time to assemble and has a long half-life (Yang, Fruh et al. 1995; Meiners, Heyken et al. 2003). The dynamic regulation of 20S proteasome activity can only be executed by means other than transcription level. Post-translational modifications provide dynamic and reversible

ways to achieve it. In fact, multiple post-translational modifications have been uncovered at 20S proteasome, including N-terminal acetylation (Coux, Tanaka et al. 1996; Bochtler, Ditzel et al. 1999), phosphorylation (Castano, Mahillo et al. 1996; Mason, Murray et al. 1998; Bose, Mason et al. 1999; Bose, Stratford et al. 2004), HNE modification (Okada, Wangpoengtrakul et al. 1999), glycosylation (Zhang, Su et al. 2003; Zachara and Hart 2004) and poly-ADP ribosylation (Ullrich, Reinheckel et al. 1999). They control proteasome assembly, subcellular distribution, activity and substrate specificity.

Postulated Principal Regulatory Sites of the 20S and 19S Subunits

20S proteasome α subunits play an essential role in 20S proteasome assembly. Besides that, it serves as a gating mechanism constituting important part of substrate selectivity and its association with different regulatory protein complexes confers proteasome plasticity (Coux, Tanaka et al. 1996; Unno, Mizushima et al. 2002). Corresponding to the multiple functions of α subunits, most reported post-translational modifications were identified from α subunits.

According to the structure of 20S proteasome, β subunits form the inner two-layer of a four-layer stacked ring structure. This dictates less solvent-exposure-surface compare to α subunits (Coux, Tanaka et al. 1996; Unno, Mizushima et al. 2002). Accordingly, less β subunit post-translational modifications were reported. Certain small molecules synthesized by microorganisms can form stable adduct with β subunits of 20S proteasome within the chamber of the stack. This is a unique modification to β subunit, and these small molecules have been using as proteasome inhibitors (Fenteany and Schreiber 1998; Kim, Myung et al. 1999; Kisselev

and Goldberg 2001; Myung, Kim et al. 2001).

The six ATPases forming the base of 19S proteasome are essential for degradation of poly-ubiquitinated proteins. They open the gate constituted by α subunits, unfold and transfer protein substrates to the proteolytic chamber. Mutational study has shown that Rpt2 is an essential part for degradation of poly-ubiquitinated proteins (Coux, Tanaka et al. 1996; Bochtler, Ditzel et al. 1999; Glickman and Ciechanover 2002). Post-translational modifications on ATPases serve as control mechanisms for their function (Mason, Murray et al. 1998; Zhang, Su et al. 2003).

Rpn10 is a poly-ubiquitin receptor, and Rpn11 is a de-ubiquitinase (UCH family). Functions of other components of 19S proteasome are less defined. Studying the post-translational control of these components would help characterize their functions.

Reported Post-translational Modifications of the 20S and 19S Subunits

Post-translational removal of N-terminal propeptide from certain β subunits ($\beta 1$, $\beta 2$, $\beta 5$, $\beta 7$) is one of the first reported post-translational modifications. This is an integral part of 20S proteasome assembly. $\beta 1$, $\beta 2$ and $\beta 5$ propeptides are removed auto-proteolytically. $\beta 7$ propeptides is removed by neighboring proteolytic active subunits. Propeptides protect N-terminal active sites from acetyl transferase before maturation and have important implication in the bias of assembly (Zwickl, Kleinz et al. 1994; Chen and Hochstrasser 1996; Seemuller, Lupas et al. 1996; Schmidt, Zantopf et al. 1999; De, Jayarapu et al. 2003).

Phosphorylation is another modification addressed by multiple manuscripts. Phosphorylated subunits were detected in the proteasome, purified from yeast and certain mammalian tissues

(Bose, Mason et al. 1999; Iwafune, Kawasaki et al. 2002). Casein kinase II (CKII) is one of 20S proteasome co-purifying enzymes, both in yeast, human erythrocytes and HEK293 (Ludemann, Lerea et al. 1993; Castano, Mahillo et al. 1996; Pardo, Murray et al. 1998). Subunits α 3, α 7 and α 6 are reported substrates of CKII. In mutagenesis studies, Ser258, Ser 263 and Ser264 of the α 7 subunit are reportedly phosphorylated in yeast; Ser243 and Ser250 of the α 7 subunit are phosphorylated in rat kidney cell line; and Ser 248 of the α 3 subunit are phosphorylated in yeast (Castano, Mahillo et al. 1996; Fernandez Murray, Pardo et al. 2002; Iwafune, Kawasaki et al. 2004). The α 7 subunit phosphorylation stabilizes the 26S proteasome ((Bose, Brooks et al. 2001). IFN- γ treatment suppresses phosphorylation of 20S proteasome subunits and decreases 26S proteasome level in the mammalian cell, simultaneously (Bose, Brooks et al. 2001; Rivett, Bose et al. 2001; Bose, Stratford et al. 2004). The effect of CKII phosphorylation at the perspective of 20S proteasome activity is less addressed. Dr. Arribas group reports that *in vitro* CKII phosphorylation of rat kidney 20S proteasome does not affect its activity (Castano, Mahillo et al. 1996); while Dr. Passeron suggests CKII phosphorylation has an impact on yeast 20S proteasome activity (Fernandez Murray, Pardo et al. 2002). PKA is another 20S proteasome co-purifying kinases identified both in kidney cell line (HEK293) and bovine pituitary. Radioactive labeling suggests two 20S proteasome subunits (27kDa and 28kDa respectively) can be phosphorylated by PKA *in vitro* ((Marambaud, Wilk et al. 1996). Moreover, PKA phosphorylation increases β 5 subunit activity of 20S proteasome *in vitro* (Pereira and Wilk 1990; Marambaud, Wilk et al. 1996). Polo-like kinase co-immuno-precipitates with 20S proteasome. Reportedly, it increases β 5

subunit activity of 20S proteasome in both *in vitro* and *in vivo* against fluorescent peptide substrate (Feng, Longo et al. 2001). Phosphorylations of 19S proteasome subunits are also reported (Mason, Murray et al. 1998; Satoh, Sasajima et al. 2001). However, the phosphorylated sites and corresponding kinases are to be further investigated. There is no previous publication that addresses endogenous protein phosphatase as part of 20S proteasome regulation mechanism.

O-linked N-acetylglucosamine modification is a form of glycosylation involved in proteasome regulation. This modification has been associated with nutritional censoring mechanism. It negatively affects the activity of 26S proteasome, while the impact on 20S proteasome activity was not significant. Rpt2, the critical subunit involved in opening the 20S gate at the bottom, is a substrate for this modification (Sumegi, Hunyadi-Gulyas et al. 2003; Zhang, Su et al. 2003; Zachara and Hart 2004).

HNE is a lipid peroxidation product, which can crosslink proteins. It inhibits 20S proteasome activity *in vitro*. The subunits are not reported (Okada, Wangpoengtrakul et al. 1999). Oxidative stress can induce different modifications upon 20S proteasome, including poly-ADP ribosylation (Davies and Goldberg 1987; Grune, Reinheckel et al. 1995; Ullrichk, Reinheckel et al. 1999; Radak, Sasvari et al. 2000). The comprehensive nature and effects of these modifications need to be further investigated and differentiated.

Regulation of Proteolytic Activities By Post-translational Modifications

As stated in the above, the regulation of proteasome activity is a collective effect of multiple post-translational tags, reflecting the impact of several control mechanisms. From the structural

and functional points of view, three types of regulations can be summarized. PTMs at 19S supervise recognition and translocation of substrate into the proteolytic active 20S proteasome chamber. PTMs at 20S proteasomes α subunits favor certain proteasome activators/repressors, or affect β subunit proteolytic activities allosterically. PTMs at 20S proteasomes β subunits regulate 20S proteasome activity, allosterically.

20S proteasome peptidase activities are not always regulated uni-directionally. Inhibitors specific to certain 20S proteasome subunit are available commercially, which supports the possibility of subunit specific activity regulation (Kisselev and Goldberg 2001; Myung, Kim et al. 2001; Kisselev, Garcia-Calvo et al. 2003). Several publications also report that proteasome activities can be controlled in a subunit specific manner in certain physiological/pathological settings *in vivo* (Andersson, Sjostrand et al. 1999; Bulteau, Lundberg et al. 2001; Basset, Raymond et al. 2002). Unfortunately, in multiple early publications, only $\beta 5$ subunit activity was assayed as a measure of overall 20S proteasome activity, which might be misleading. All three 20S proteasome subunit specific substrates have to be used to evaluate its overall functionality.

Clinical Implications of Proteasomal Function

Proteasomal Dysfunction Associated with Diseased-Phenotypes

Consistent with the large variety of proteins turned-over by the proteasome, malfunctioning of this complex can result in an ever growing list of diseases. Cancer is among the first and the most important proteasome malfunction-associated diseases reported. To date, a tremendous amount of studies have been conducted into the role of the proteasome in cancer. Bortezomib

(previously PS-341) from Millennium Pharmaceuticals has been used in cancer therapy (LeBlanc, Catley et al. 2002; Orlowski, Stinchcombe et al. 2002; Chauhan, Li et al. 2003). The involvement of Ubiquitin-proteasome system in cancerous pathogenesis is complicated. Degradation of pro-apoptotic protein, caspases, is controlled by proteasome (Dallaporta, Pablo et al. 2000; Chen, Smith et al. 2003). Degradation of p53 and other essentially cell cycle proteins are also manifested by the proteasome (Higashitsuji, Higashitsuji et al. 2005; Richardson, Mitsiades et al. 2005).

The involvement of the proteasome in neural degenerative diseases is well recognized, such as Alzheimer's disease and Parkinson's disease (Glickman and Ciechanover 2002; Dawson and Dawson 2003; Ross and Pickart 2004). In fact, parkin is an E3. The Liddle syndrome is the result of a mutation at kidney ENaC channel protein, which prevents its turnover (Glickman and Ciechanover 2002; Debigare and Price 2003). A mutation at an E3 (E6-AP) occurs in the Angelman syndrome. Ubiquitin-proteasome system disorder also affects immuno-response and muscle wasting (Glickman and Ciechanover 2002).

Postulated Effects of Proteasomes in Ischemic Injury and Protection

Recently, the effect of proteasome inhibition in cardiac protection came to cardiologist's attention. Dr. Kukan recently published a review article (Kukan 2004); in which it stated ischemia reperfusion induced infarction in brain, heart, kidney and liver can be minimized by application of proteasome inhibitors. Several research papers also support the view that inhibition of proteasome is a benefit against cardiac infarction upon ischemia/reperfusion insult (Campbell, Adams et al. 1999; Zhang, Zhang et al. 2001; Luss, Schmitz et al. 2002; Stangl, Gunther et al. 2002; Pye,

Ardeshirpour et al. 2003).

Though there have been consistent reports of proteasome inhibition leading to protection against ischemia, the mechanism remains unknown due to multiplicity of cell types in a target tissue and proteasome involves in multiple cell biological pathways. Nevertheless, several hypotheses have been proposed.

The first hypothesis is the reduction of immuno-response. In this hypothesis, proteasome inhibition downregulates NF- κ B pathway in endothelial cells and lymphatic cells. Consequently, less lymphatic cells attach and filtrate through micro-vessels formed by endothelial cells, which blocks blood flow. On the other hand, this downregulation reduces the release cytotoxic cytokines and inflammatory factors. The effect of NF- κ B downregulation in cardiomyocyte is unfortunately not addressed (Campbell, Adams et al. 1999; Elliott, Zollner et al. 2003; Pye, Ardeshirpour et al. 2003).

Secondary up-regulation of heat-shock proteins (HSPs) after proteasome inhibition is another proposed hypothesis. Over-expression of HSPs is well recognized to be cardioprotective. However, in this specific setting, whether this upregulation is the primary protective mechanism or just a side-effect, needs further investigation. There is another complicating factor. Several groups report oxidized proteins, due to free radicals produced in ischemia/reperfusion injury, are preferably degraded by 20S proteasome independent of ubiquitination. The increased ability in preventing protein denaturing by HSPs and the decreased ability in turning-over irreversibly oxidative damaged proteins must be balanced (Kim, Kim et al. 1999; Luss, Schmitz et al. 2002;

Stangl, Gunther et al. 2002; Aki, Yoshida et al.2003).

The proteasome is involved in the control of apoptosis-related proteins (such as caspases and p53). The role of this control in the proteasome inhibited cardioprotective model need to be studied (Dallaporta, Pablo et al. 2000; Chen, Smith et al. 2003; Higashitsuji, Higashitsuji et al. 2005; Richardson, Mitsiades et al. 2005). There are more hypotheses that can be formed, making this model even more complicated.

In short, proteasome-inhibition-induced cardio-protection involves the interplay between different cell types and candidate proteins. Simplified models need to be established to understand the molecular details of this process and help design more specific pharmacological reagents.

Hypotheses And Aims of This Study

Hypotheses

With information gained from the yeast proteasomes combined with our current understanding and postulation of the mammalian proteasomes, we have formulated the following testable hypotheses:

Hypothesis I.

The Murine Cardiac 20S Proteasomes Are A Collection Of Multiprotein Complexes; This Sub-organelle Is Composed Of Essential Subunits And Key Ancillary Associating Partners.

Hypothesis II.

The 20S Proteasomes Hold Multiple Regulatory Sites That Are Essential To The Modulation of Cardiac Proteolytic Activity. Posttranslational Modification (e.g., Phosphorylation) Of The 20S Subunits Contributes To This Regulatory Process.

Hypothesis III.

The Associating Partners of The 20S Proteasomes Facilitate/Assist Isoform-Selective Modulation of The Cardiac Proteolytic Activity.

Aims Of This Study

To test the above hypotheses, we have organized the following specific aims.

Specific Aim I.

Purification, Isolation, And Proteomic Characterization Of The Murine Cardiac 20S Proteasomes.

Specific Aim II.

Characterization Of Key Phosphorylation Events Of The 20S Proteasomes; Determination Whether Such Posttranslational Modifications Play Important Roles In The Modulation of Cardiac Proteolytic Activity.

Specific Aim III.

Identification And Characterization Of The Associating Partners of The 20S Proteasomes; Determination Of Their Roles In The Modulation Of Cardiac Proteolytic Activity.

CHAPTER II

PURIFICATION OF 20S PROTEASOME FROM MURINE HEART

Introduction

The setup of a reproducible large-scale purification protocol is the first critical step to systematically study the structural and the functional characters of murine cardiac 20S proteasome. Dr. Avram Hershko, Dr. Aaron Ciechanover from Israel and Dr. Irwin Rose from United States of America pioneered the studies in ubiquitin-proteasome degradation pathway. As recognition of their contributions, they shared the Nobel Prize in Chemistry 2004. The highlight of their contribution is elucidation the mechanism of the selective ubiquitination and discovery of E1, E2, and E3 in the 80s, which brought controlled protein degradation to the focus of the biological community. To date, the proteasome is found in all three kingdoms of organisms: archbacteria, eubacteria and eukaryotes (Coux, Tanaka et al. 1996; Bochtler, Ditzel et al. 1999; Glickman and Ciechanover 2002). The primary sequence and quaternary structure of the 20S proteasome show great conservation across species. Several purification strategies have been proposed. Based on the nature of the methods, they can be classified into three groups: classic biochemical purification protocols (referred to as the classical method from now on), immunoprecipitation protocols (referred to as the immunoprecipitation method from now on) and affinity purification protocols (referred to as the affinity method from now on) (Glickman and Ciechanover 2002; Leggett,

In the classical protocol, we purified the 20S proteasome according to its unique biochemical properties, including high stability and solubility in high salt solution, high molecular weight and charge status at certain pH. Sample prepared by the classical method has the advantage of high reproducibility, high purity and the possibility of high yield. The disadvantages include a longer purification time (3-4 days to finish). Proteins loosely associating with 20S proteasome may be lost during the procedure. The immunoprecipitation (IP) protocol is usually carried out by IP with proteasome subunit-specific antibodies. The IP procedure is less time-consuming and offers the possibility to purify a population of endogenous proteasome complexes. However, a simple IP procedure cannot match a stringent purity requirement. The IgG light chain has a similar molecular weight as some of the 20S proteasome subunits, which makes mass spectrometric study difficult. It is also economically prohibitive to use this method for large-scale purification. The affinity protocol combines the advantages of the two previous approaches. It provides an economical way for large-scale, high purity, high reproducibility proteasome purification in short period of time. The choice of affinity tags is diverse. FLAG, 6X His, protein A have all been proved to be applicable (Tongaonkar, Chen et al. 2000; Leggett, Hanna et al. 2002; Iwafune, Kawasaki et al. 2004). However, the need to transfect cells with plasmids that would express the tagged protein limits the current applications to the cell lines and unicellular organisms (yeast, etc). The choice of protocol depends on the purpose of the study, nature of the sample and the scale of purification needed.

20S proteasome has a molecular weight between 600-700kDa, depending on the species. It is stable in high salt, non-ATP solution. On the other hand, 26S proteasome, with a molecular weight ranging from 1300kDa to 2100kDa (19S-20S or 19S-20S-19S), dissociates in such a

solution into 20S proteasome and other protein complexes in the presence of high salt (Eytan, Ganoth et al. 1989; Ugai, Tamura et al. 1993; Fischer, Hilt et al. 1994).

Existing Protocols To Purify Proteasomes From Yeast

Yeast is a unicellular eukaryotic organism that can be genetically manipulated easily and expended in large-scale. All three types of protocols mentioned above are applicable to this organism.

In term of classical purification, there are two representative procedures that are very similar in nature. One, as described in *methods in molecular biology* (Leggett, Glickman et al. 2005), applies yeast cytosolic proteins to two consecutive anion chromatographies followed with gel filtration chromatography. In the other procedure (Groll, Ditzel et al. 1997), yeast 20S proteasome are purified through anion ion-exchange chromatography, hydroxy-apatite chromatography and gel filtration chromatography sequentially. The presence of ATP in the purification buffers greatly affected the end product obtained. Including ATP in the buffer resulted in both singly capped and doubly capped 26S proteasome, with the second form usually dominant. When buffers free of ATP are utilized, the 20S proteasome isolated from yeast was of a purity that the 20S could be crystallized.

With the power of genetic manipulation, more and more investigators turn to use affinity resins to bail affinity tagged yeast proteasome (Tongaonkar, Chen et al. 2000; Leggett, Hanna et al. 2002; Iwafune, Kawasaki et al. 2004). This approach significantly simplifies purification procedure, shortens the time needed and lowers the harshness of purification procedure. This preserves

salt-labile proteasome subunit or proteasome associating proteins, which would have been lost during classical procedure.

Existing Protocols To Purify Proteasomes From Mammalian Cell Culture

There are two sets of protocols for purifying 20S proteasome from mammalian cells, based on the nature of the starting material.

For mammalian cell lines, there is no report on large-scale 20S proteasome purification. It would be time-consuming and cost-prohibitive to pursue such procedure. IP and affinity purifications are the methods of choice. IP is a simpler procedure without the needs of transformation. However, the introduction of antibody in IP procedure limits the maximal purity we can reach. The affinity chromatography promises a quicker purification procedure and the potential of reaching higher purity. On the other hand, the transformation procedure would affect the status of the cells and affinity-tagged proteasome might have acquired distinct properties compared to the endogenous counterparts. For tissue samples, in most cases, the quantity of the sample to start with is not the major concern. On the other hand, genetic manipulation needs transgenesis, which is a challenging and risky task. To date, the majority of mammalian 20S proteasome studies are done with preparations from reticulocyte, liver, and skeletal muscle, due to their easy availability and accessibility (Rivett 1985; Hough, Pratt et al. 1987; Hoffman, Pratt et al. 1992; Castario, Mahillo et al. 1996; Thomas, Oosthuizen et al. 2002; Hayter, Doherty et al. 2005). In the classical procedure, 20S proteasome is purified step by step according to its unique biochemical properties: solubility at high salt concentration (ammonium precipitation), charge state

(retention time through an anion ion exchange column) and high molecular weight (ultra-centrifugation or gel-filtration chromatography). There are some applications of the IP procedure, which are small scale and just used to study one or two aspects of 20S proteasome (Shibatani and Ward 1995; Feng, Longo et al. 2001).

Significance of Purifying Proteasomes From Cardiac Tissues

The proteasome has been reported to play important roles in cardiac physiology and pathology, especially in ischemia/reperfusion condition (Campbell, Adams et al. 1999; Bao, Sato et al. 2001; Pye, Ardeshirpour et al. 2003; Townsend, Cutress et al. 2004). A comprehensive understanding of the cardiac 20S proteasome structure, function and regulation is the pre-requirement to understand its role in biological context. To gain such knowledge, a highly reproducible and large-scale purification protocol has to be established.

The mouse is an indispensable animal model to study cardiac physiology due to its high reproduction rate, availability of well-established pathological models, and transgenic lines. However, the use of mice also imposed challenges. The size of mouse heart is small (around 0.15g for an 8 week old mouse heart). Moreover, proteasome expression level in cardiac tissue is lower than that of liver tissue. These arbitrate a criterion to exam the success of purification protocol: The loss of proteasomes during purification has to be minimized to not be cost-prohibitive. Large-scale murine cardiac 20S proteasome purification protocol has not been reported. Murine cardiac 20S proteasome purification protocol was able to purify 800 μ g of >95% pure proteasome from 12g of cardiac tissue in a single run. This protocol was optimized from a liver purification protocol (French, Mayer et al. 2001) to fit the nature of cardiac sample and gain higher purity.

Materials and Methods

The Use of Mouse Lines

8 week old male ICR mice from Harlan were used in all studies. Mice were euthanized according to NIH and UCLA DLAM guidelines. Heart tissue was taken, washed with TBS to remove blood and frozen in -80°C freezer until used.

Isolation of Cytosolic Fraction from Whole Heart Lysate

12g of mice heart tissue was homogenized with glass potter homogenizer in homogenize buffer (20mM Tris-base pH 7.8, 0.1mM EDTA, 1mM DTT, supplemented with protease inhibitor cocktail from Roche and phosphatase cocktails from Sigma). Homogenate was forced through 4 layers of gauge to remove unbroken connective tissues. Cytosolic fraction was collected as the supernatant after 2hr centrifugation at 25,000g.

The Use of Protease and Phosphatase Inhibitors

Protease inhibitor cocktail (complete, EDTA free) was acquired from Roche. 1 tablet was used in 50ml solution. It was essential to minimize the non-specific degradation of proteins by lysosomal proteases. Importantly, this cocktail does not inhibit 20S proteasome activities.

Phosphatase inhibitor cocktail 1 (serine, threonine phosphatases inhibitors in DMSO) and cocktail 2 (acid, alkaline and tyrosine phosphatases inhibitors in aqueous solution) were acquired from Sigma Aldrich. Both cocktails are supplied as 50X stock. Use of both inhibitors was important to preserve the endogenous phosphorylation state of intracellular proteins during purification.

Proteasome Activities Assay and Inhibitors

10 μ l of 10X proteasome assay buffer (250mM HEPES, pH 7.5; 5mM EDTA; 0.3% SDS) was aliquotted in each well of a 96-well microplate. 70 μ l water, 10 μ l of proteasome sample and 10 μ l of 10X fluorophore-linked peptide substrates (500 μ M Suc-Leu-Leu-Val-Tyr-AMC, Bachem) were used sequentially to each well establishing the assay mixture. The incubation time was set to 1hr after which the activity was measured by fluorometer (Fluoroskan Ascent, Thermo Electron) at an excitation wavelength of 390nm and an emission wavelength of 460nm.

Fast-Pressure Column Chromatography (FPLC)

All FPLC were conducted using the AKTA Purifier (GE Healthcare, formerly Amersham). There are two anion ion-exchange chromatography steps during the purification procedure: preparative scale chromatography and analytical scale chromatography. Same receipt of buffer A and buffer B were used for both procedures.

Buffer A: Tris 20mM pH 7.4, MgCl₂ 5mM, DTT 0.5mM, Glycerol 10%;

Buffer B: Tris 20mM pH 7.4, MgCl₂ 5mM, DTT 0.5mM, Glycerol 10%, KCl 600mM.

Preparative chromatography: 200ml Q Sepharose Fast Flow resin (from GE healthcare) was packed in XK 26/40 column (from Amersham) in-house. Sample was loaded through a 10ml Superloop. Then its components were resolved with 3-step stepwise salt gradient elution at flow rate 5ml/min: 45% B, until UV280nm monitor reading goes to baseline; 75% B, collect 200ml; 100% B, until UV280nm monitor goes to baseline.

Analytical chromatography: Pre-packed Mono Q HR 5/50 column was acquired from GE

healthcare. Sample was loaded through a 2ml sample loop. Then it was resolved with a linear salt gradient from 0% to 100% B within 17.5 column volume (CV equals 1ml).

SDS-PAGE and Western-blotting

SDS PAGE was performed with Bio-Rad Mini-Protean II apparatus according to classic Laemmli protocol using 12.5% poly-acrylamide gels. Electrophoresis was conducted at 120V DC for 1hr. After that, the gel could either be visualized by Colloidal Coomassie blue G-250 staining protocol or transferred to nitrocellulose membrane (transblot) for western-blotting. Transfer efficiency was evaluated by Ponceau S staining.

Western-blotting was proceeded according to standard chemiluminescent procedure: The transblot was blocked with 5% milk for 1hr; incubated with 1st antibody (1000X dilution in 5% milk, 1% Tween-20) for 1hr; Wash with TTBS (Tris-buffered solution with 1% Tween-20) for 3×5 min; incubated with HRP-linked 2nd antibody (3000X dilution in 5% milk, 1% Tween-20) for 1hr; Wash with TTBS for 3×5 min; Finally the transblot was incubated with enhanced chemiluminescence (ECL) reagent (GE healthcare) for 1min and chemiluminescent signal recorded with film (Kodak).

2D Electrophoresis and Western-blotting

2D electrophoresis was conducted using a Bio-Rad 11cm apparatus. Firstly, purified 20S proteasome was desalted by TCA/Acetone precipitation. Desalted dry pellet was resuspended and resolubilized with IPG rehydration buffer (7M urea, 50mM DTT, 4% CHAPS, 0.2% 3-10 Bio-Rad ampholytes). 11cm NL (non-linear) (BioRad) IPG was rehydrated in the IPG rehydration solution overnight, and then isoelectrofocusing was conducted with Bio-Rad IEF cell (250V, linear

gradient 20 min, 250V, step and hold 5hrs, 3000V, linear gradient 1hr, 3000V, step and hold 1hr, 8000V, linear gradient 1hr, 8000V, step and hold for a total of 49375 Vhrs.). After IEF, proteins in IPG strips were reduced by 2% DTT solution and alkylated by 2.5% IAA sequentially for 10 minutes each. The second dimension electrophoresis was done using Bio-Rad pre-cast Criterion gel (12.5%). The resulting 2D gel will be either stained with SYPRO RUBY or transferred to nitrocellulose membrane, which is ready to be used in western blotting.

Western-blotting (WB) was carried out either according to the standard chemiluminescent procedure (described in II.B.6) or another fluorescent procedure using the Odyssey. Odyssey fluorescent scanner (Licor) was used to fluorescent WB: Block the transblot with 1% gelatin for 1hr; Probe with 1st antibody (1000X dilution in 1% gelatin, 0.5% Tween-20) for 1hr; Wash with TTBS (Tris-buffered solution with 1% Tween-20) for 3×5 min; Probed with Fluorophore-linked 2nd antibody (3000X dilution in 1% gelatin, 0.5% Tween-20) for 1hr; Wash with TTBS for 3×5 min; Finally the transblot was scanned. Scanned images were overlaid with SYPRO ruby stain image of the counterpart.

HPLC Coupled Tandem Mass Spectrometry

Proteins resolved by electrophoresis were digested with trypsin (Promega) and extracted from the gel. The tryptic peptides were resolved with RP-HPLC column coupled online to a mass spectrometer, which identified proteins have been digested. Three types of mass spectrometers have been used in this study: Q-STAR (Applied Biosystems, Q-Tof), LCQ Deca XP (Thermo Electron, Ion-trap) and LTQ (Thermo Electron, linear ion-trap). All setups were conducted with LC

flow rate at 200nl/min and mass spectrometer set at data-dependent-acquisition mode. Spectra acquired were searched against IPI mouse database using MASCOT search engine (Matrix Science). Only proteins identified with more than two peptides (peptide score higher than 25 each) and protein score higher than 80 were reported as positive.

Electron-microscopic Study of Murine Cardiac 20S Proteasome

Carbon-coated copper grid was positively charged. 10 μ l purified murine cardiac 20S proteasome at 0.15 μ g/ μ l in 20mM Tris-HCl (pH 7.5) was allowed to bind to the grid for 15min. The grid was washed with 20mM Tris-HCl (pH 7.5) and then stained with 4% uranic acid (depleted) for 2min. Stained grid was left dry for 15min before ready for electro-microscopic (EM) study. Murine cardiac 20S proteasome images were recorded with film.

Summary Of the Salient Steps/Reagents That Assured Successful Purifications

Introduction of protease and phosphatase inhibitor cocktails were important to maintain 20S proteasome in its endogenous state. All purification steps were conducted at 4°C, 10% glycerol was included in all buffers to minimize denaturation and inactivation of 20S proteasome during purification. Ultra-centrifugation is used to separate high molecular weight protein complexes from the rest of the sample instead of using gel-filtration chromatography. This would reduce the loss of 20S proteasome at the expense of longer separation time.

Results

The Expression of 20S Proteasome In the Murine Heart Versus That In The Liver

20S proteasome has been purified from liver (French, Mayer et al. 2001). The expression level of the 20S proteasome in the heart and liver showed the 20S proteasome level in the heart is significantly less than that of the liver (Figure 1).

Flow-Chart of Murine Heart 20S Proteasome Purification

20S proteasome was reproducibly purified on large-scale from murine cardiac tissue according to its biochemical properties: high stability and high solubility at high salt concentration (ammonium sulfate precipitation); unique charging properties at certain pH (anion ion-exchange chromatography) and high molecular-weight (ultra-centrifugation). The detailed protocol is shown in a flowchart (Figure 2). This protocol reproducibly gained 20S proteasome with purity higher than 95% from murine heart.

Chromatographic Purification Steps

There were two anion ion-exchange chromatographic steps in the purification procedure.

In preparative scale ion-exchange chromatography, pooled murine cardiac homogenate cytosolic fraction was resolved through a 200ml column (total volume). 3-step salt concentration gradient elution resulted in 3 peaks detected with UV280nm monitor. The first peak was the flow through fraction, which represented contaminant proteins with less negative charges at pH 7.5. The second peak enriched with murine cardiac 20S proteasome. The last peak enriched with RNAs and highly negative charged proteins at pH 7.5.

In the final analytical chromatography, sample was resolved over a 1ml column with a linear salt concentration gradient from 0% B to 100% B (0mM KCl to 600mM KCl). 20S proteasome was recovered in fractions at around 60% B (360mM KCl), which were collected at fraction size of 0.3ml. According to the UV280nm of chromatogram, 20S proteasome is well separated from other proteins, resulting in purity higher than 95%.

SDS-PAGE and Western-Blot Identification of 20S Proteasome Enriched Fractions

Fractions enriched in murine cardiac 20S proteasome peak, suggested by UV280nm chromatogram, were resolved by SDS-PAGE in duplicate. One replica was stained with Coomassie blue G-250 to visualize total proteins in the gel (Figure 4, Panel A). It reproduced characteristic pattern of 20S proteasome, in which multiple bands corresponding to 20S proteasome subunits were packed between 20kDa and 30kDa. Western-blot over the other replica using antibodies specific to multiple 20S proteasome α subunits, confirmed the murine cardiac 20S proteasome were enriched in the same fractions (Figure 4, Panel B) as indicated in Coomassie stain.

The Purified 20S Proteasome is Proteolytically Active

Fractions enriched with murine cardiac 20S proteasome were aliquotted and stored at -80°C. 20S proteasome β 5 subunit activity assay was conducted over these fractions. The activity curve gained (Figure 5) was consistent with UV280nm, Coomassie stain and western-blot. This documented that these fractions were indeed enriched with murine cardiac 20S proteasomes and they were catalytically active.

Electron-microscopic (EM) images of murine cardiac 20S proteasome revealed that they were intact. The bottom and the side view of the protein complex under EM were consistent with the theoretical shape gained from X-ray crystallography study (Figure 7).

2D Electrophoresis and LC-MS/MS Characterization of The Molecular Components In The Murine Cardiac 20S Proteasomes

Murine cardiac 20S proteasome preparation was resolved by 2D electrophoresis and then stained with SYPRO ruby. All major spots in the 2D gel were sequenced with LC/MS/MS to gain protein IDs. All 20S proteasome constitutive subunits and one inducible subunit were identified (Figure 6). The other two inducible subunits were identified in a parallel 1D SDS-PAGE and LC/MS/MS study (data not shown). Summary of murine cardiac 20S proteasome LC/MS/MS data is shown in Table 1.

Theoretical 2D map of murine cardiac 20S proteasome and yeasts 20S proteasome was generated (Figure 6). Theoretical isoelectric points and molecular weights were obtained using bioinformatic tools available from the Swiss Institute of Bioinformatics (www.expasy.org).

2D Electrophoresis and Western-blotting Reveals Post-translation Modifications on Multiple 20S Proteasome Subunits

2D electrophoresis followed by immunoblotting suggested certain type of PTMs (phosphor-threonine, phosphor-serine, phosphor-tyrosine) occurred endogenously at multiple 20S proteasome subunits (Figure 8, Panel A). Phospho-threonine specific antibody (Santa Cruz)

recognized α 1, 6 and β 3, 7 subunits. Phospho-serine specific antibody (Zymed) recognized α 1, 6, 7 and β 2, 3, 7 subunits. Phospho-tyrosine specific antibody (Santa Cruz) recognized 1,2,6 subunits, and 3, 7 subunits (Figure 8, Panel A).

Serine phosphorylation of α 7 subunits was confirmed with mass spectrometry. The phosphorylation site was identified to be C-terminal serine-249 residue.

1D Electrophoresis and LC-MS/MS Analysis Identifies Critical 20S Proteasome Associating

Partners

Comprehensive proteomic study was conducted over purified murine cardiac 20S proteasome with high throughput LC-MS/MS sequencing. This uncovered casein kinase II (CK2), cAMP-dependent protein kinase (PKA) and protein phosphatase 2A (PP2A) (Figure 9) as 20S proteasome associating partners (Table 2).

Discussion

20S Proteasomes Can Be Purified as An Intact Complex from Murine Heart

20S proteasome is protein complex with unique properties, which can be used to isolate it from a complex mixture such as tissue homogenate. It has high molecule weight, high stability and solubility at high salt concentration and high affinity to anion ion-exchange resin at pH 7.5. In a classical biochemical purification procedure, these unique properties facilitate its purification. In SDS-PAGE and 2D gel electrophoresis, subunits of purified murine cardiac 20S proteasome showed the same stoichiometry, which indicated 20S proteasome could be purified from murine heart intact. On the other hand, Coomassie blue stain showed the absence of 26S proteasome. Electro-microscopic images gained from further analysis of the purification preparation provided unquestionable evident that 20S proteasome was intact and 26S proteasome was absent.

The Purified 20S Proteasome is Proteolytically Active

The 20S proteasome has two copies of three catalytic active subunits ($\beta 1$, $\beta 2$ and $\beta 5$) with distinct peptidase activities (Caspase-like activity, trypsin-like activity and chymotrypsin-like activity, respectively). Recombinant peptides with covalent-linked fluorophore (Z-Leu-Leu-Glu-AMC, Bz-Leu-Ser-Thr-Arg-AMC, and Suc-Leu-Leu-Val-Tyr-AMC) were used to assay 20S proteasome activities (Caspase-like activity, trypsin-like activity and chymotrypsin-like activity, respectively). Besides substrate specificity, there are three more characteristics that distinguish eukaryotic 20S proteasome from other proteinases/peptidases. It has unique high molecular weight; it is endogenously in a latent form and this latency can be relieved biochemically with detergent *in vitro*; It can be specifically inhibited by inhibitors (epoxomicin and Lactacystin) designed to inhibit N-terminal hydrolase (Ntn) family proteinase. 20S proteasome purified from murine heart was shown to be enzymatically active.

Differential 20S Proteasome Complexes Co-exist in Murine Heart

As shown from the LC-MS/MS sequencing study, both constitutive and inducible 20S proteasome subunits existed in the murine cardiac purification preparation. Moreover, SYPRO Ruby staining and Western blot of murine cardiac 20S proteasome resolved with 2D gel indicated the presence of endogenous PTMs on 20S proteasome subunits. This gave a sense on the complexity of 20S proteasome population in murine heart, which suggested this mega-protease is under the joint control of multiple mechanisms. Despite a variety of PTMs, phosphorylation is the most interesting due to its ubiquitous and dynamic nature. A comprehensive proteomic study combining the power of western blot with specific antibodies and mass spectrometry, showed that phosphorylation is an important regulator of the 20S proteasome activity endogenously.

20S Proteasome Associating Partners May Play Regulatory Roles

Elucidated by the purification flow chart (Figure 2), the whole purification is a three-day procedure including one salt precipitation, one dialysis, one ultra-centrifugation and two ion-exchange chromatography steps. The purity of 20S proteasome in the final preparation is higher than 95%. In this stringent condition, only proteins that associate with 20S proteasome fairly strong would have remain in the final preparation. In fact, the well-known 20S proteasome stoichiometric associating partner, the 19S, cannot survive this procedure. 20S proteasome associating proteins consistently identified from the purification preparation should have significant impact on 20S proteasome functions. Indeed, several proteins have been consistently identified in the purification preparation and correspond to previous reports. Among them, PKA, CK2 and PP2A are kinases or phosphatase that regulate protein phosphorylation status and possibly responsible for endogenous 20S proteasome phosphorylation control.

Summary

Large scale 20S proteasome purification from murine heart was established according to classic biochemical protocol. There were optimizations within the chromatography steps and ultra-centrifugation step. Murine cardiac tissues were more precious than rat liver tissue and 20S proteasome expression level was lower in the heart than that of the liver, these optimizations have been proved to be important. In the final chromatography step, 20S proteasome was recovered as a single peak without overlapping with others. The purity of the preparation was further evaluated by multiple methods. Staining of purified murine cardiac 20S proteasome resolved with SDS PAGE or 2D electrophoresis documented the purity higher than 95%. Electronic-microscopy image showed the shape of 20S proteasome in the preparation was consistent with that documented in X-ray study, which served as a measure of the integrity of 20S proteasome in the preparation. Proteasome activity assay proved its activity had been preserved during the preparation procedure.

In a proteomic study of purified murine cardiac 20S proteasome, the complexity of this seemingly simple protein complex emerged. Besides all 14 constitutive expressing subunits, inducible subunit $\beta 5i$ was also identified within the 2D gel. The other two inducible subunits, $\beta 1i$ and $\beta 2i$, were identified by mass spectrometer, after resolving purified murine cardiac 20S proteasome with SDS PAGE. In addition, several 20S proteasome subunits were each represented by multiple spots in the 2D gel, which was a clear indication of the existence of PTMs. Consistent to this notion, the pI of purified murine cardiac 20S proteasome was 5.2, which is 0.8 unit lower than the value predicted with bioinformatic tools for un-modified 20S proteasome. In a

subsequent western blot study, these PTMs appear to at least include serine-phosphorylation, threonine phosphorylation and tyrosine phosphorylation. $\alpha 7$ subunit was identified to be phosphorylated at serine-249 by mass spectrometry.

Several enzymes, regulating 20S proteasome phosphorylation status, have been identified with purified cardiac 20S proteasome preparation, namely, PKA, CK2 and PP2A. PKA and CK2 have been identified in other tissue as 20S proteasome associating proteins, while PP2A has been suggested as potential 20S proteasome associating protein by a yeast-2-hybrid experiment. The functional impacts of these enzymes on 20S proteasome are discussed in detail in the following section.

Figure 1. The Expression of 20S Proteasome In the Murine Heart and Liver. Murine heart and liver cytosolic proteins were resolved by SDS-PAGE. Western blot was conducted using antibody that recognize multiple 20S proteasome α subunits (Biomol). 20S proteasome expression level in the heart is much lower than that of liver (absorption density analysis showed heart 20S proteasome expression level is 1/3 of that of liver).



Figure 2. Flow-Chart of Murine Heart 20S Proteasome Purification. Murine cardiac 20S proteasome purification is a classical biochemistry purification procedure, which purify the 20S proteasome according to its biochemical property.

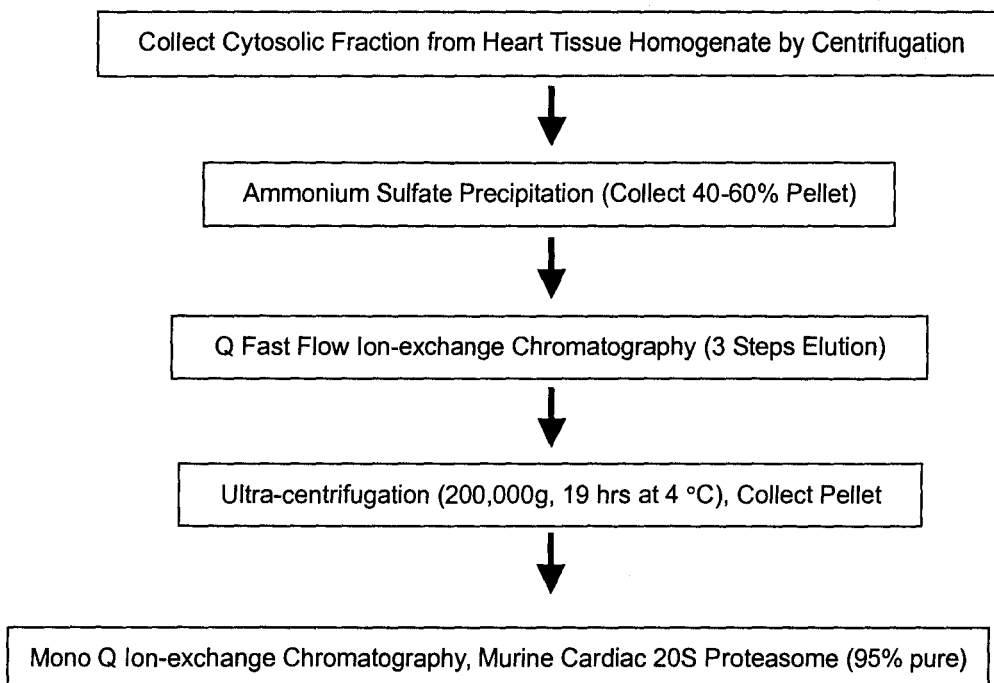
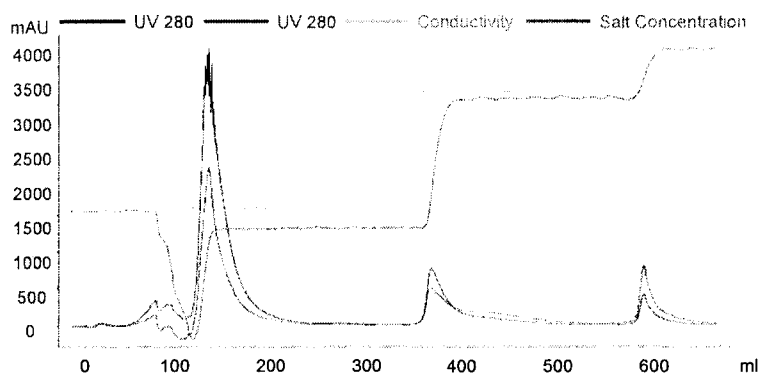


Figure 3. Chromatographic Purification Steps. Panel A. Preparative scale Q Sepharose Fast Flow anion ion exchange chromatogram. 3 steps salt concentration was used. 45% B (270mM KCl) to let majority of contaminant protein flow through the column without binding; 75% B (45mM KCl) to elute semi-pure 20S proteasome from the column; 100% B (600mM KCl) to remove RNA bound to the column. **Panel B.** Analytical scale Mono Q HR 5/50 anion ion exchange chromatogram. Linear salt concentration gradient was used to resolve proteins in the sample. All the peaks (UV 280nm) were well separated and 20S proteasome was represented with the highest absorbance.

Panel A.



Panel B.

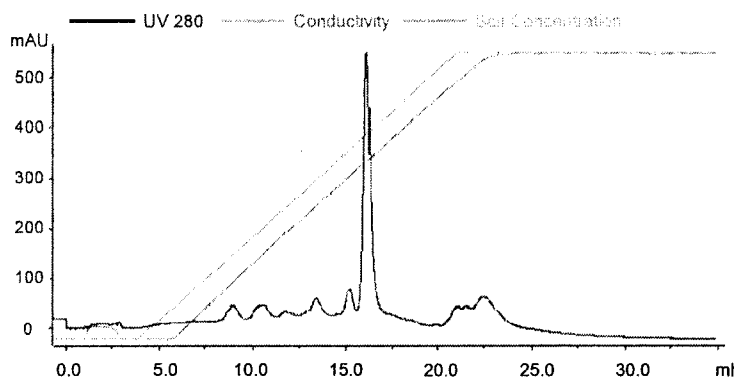
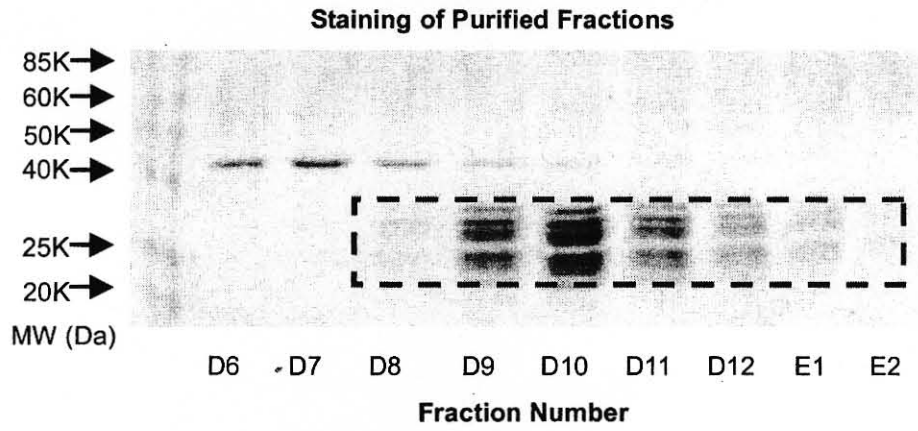


Figure 4. SDS-PAGE and Western-Blot Identification of 20S Proteasome Enriched Fractions.

Panel A. Fractions collected by analytical Mono Q FPLC were resolved by SDS-PAGE and then stained with coomassie blue. 20S proteasome subunits have unique pattern in SDS-PAGE, in which multiple bands clustered between 20KDa and 30KDa. The proteasome-enriched fraction shown in SDS-PAGE Coomassie stain was consistent with UV280 chromatogram. **Panel B.** Same fractions collected by analytical Mono Q FPLC were resolved by SDS-PAGE and transferred to nitrocellulose membrane. Western blot was conducted with antibody recognizing multiple 20S proteasome α subunits (α 1, 2, 3, 5, 6, 7). The proteasome-enriched fraction shown in western blot was consistent with both UV280 chromatogram and Coomassie blue staining pattern.

Panel A.



Panel B.

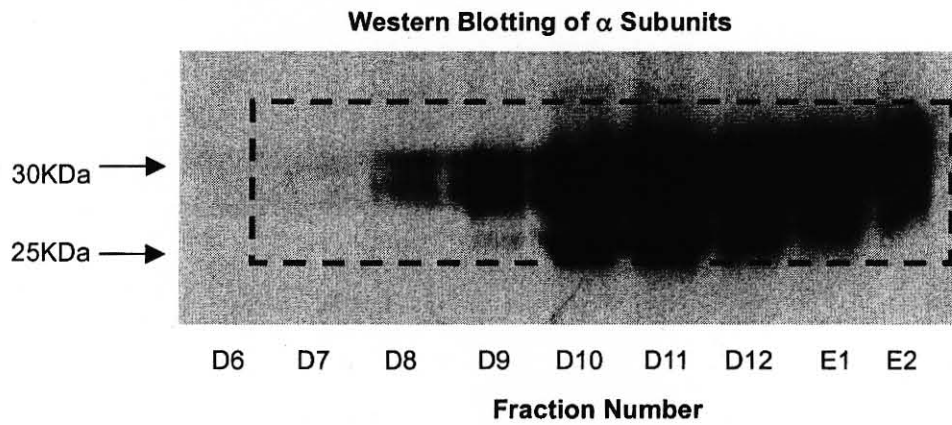


Figure 5. The Purified 20S Proteasome is Proteolytically Active. 20S proteasome β 5 subunit activity was measured with fractions collected by analytical Mono Q FPLC. The proteasome-enriched fraction shown in the assay was consistent with UV280 chromatogram, Coomassie stain and western blot. This suggested that purified 20S proteasome from murine heart are catalytically active.

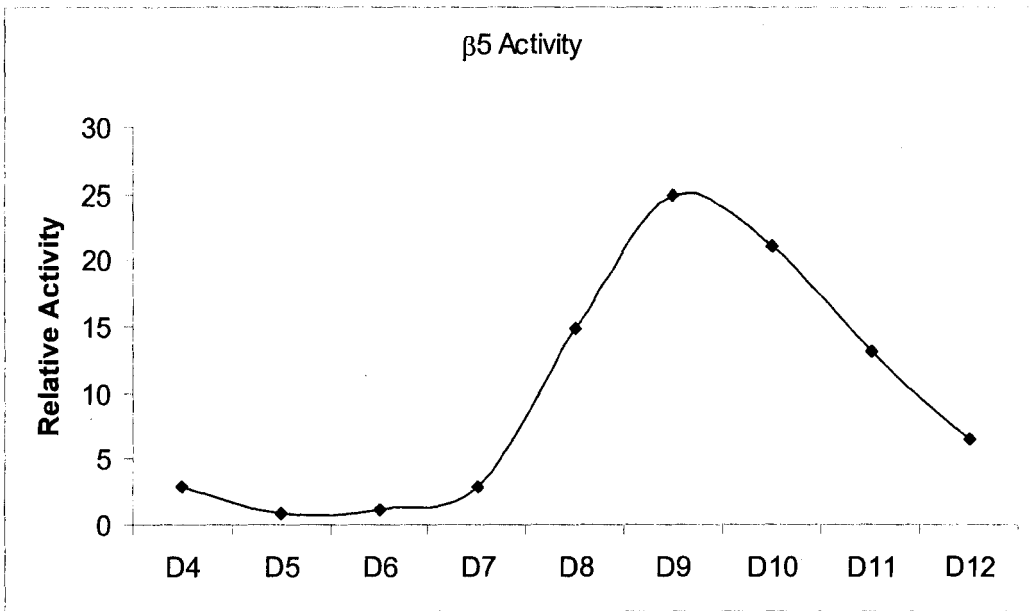


Figure 6. 2D Electrophoresis and LC-MS/MS Characterization of The Molecular Components In The Murine Cardiac 20S Proteasomes. Panel A. Theoretical 2D map of murine 20S proteasome. Isoelectric point and molecular weight of 20S proteasome subunits were predicted with bioinformatic tool (www.expasy.org). **Panel B.** SYPRO Ruby stain of murine cardiac 20S proteasome resolved by 2D Electrophoresis. Spots from the 2D gel were picked and identified with mass spectrometer. Several 20S proteasome subunits were represented by multiple spots in the 2D gel, which is a clearly indication of post-translational modification. **Panel C.** Theoretical 2D map of murine 20S proteasome. Isoelectric point and molecular weight of 20S proteasome subunits were predicted with bioinformatic tool (www.expasy.org).

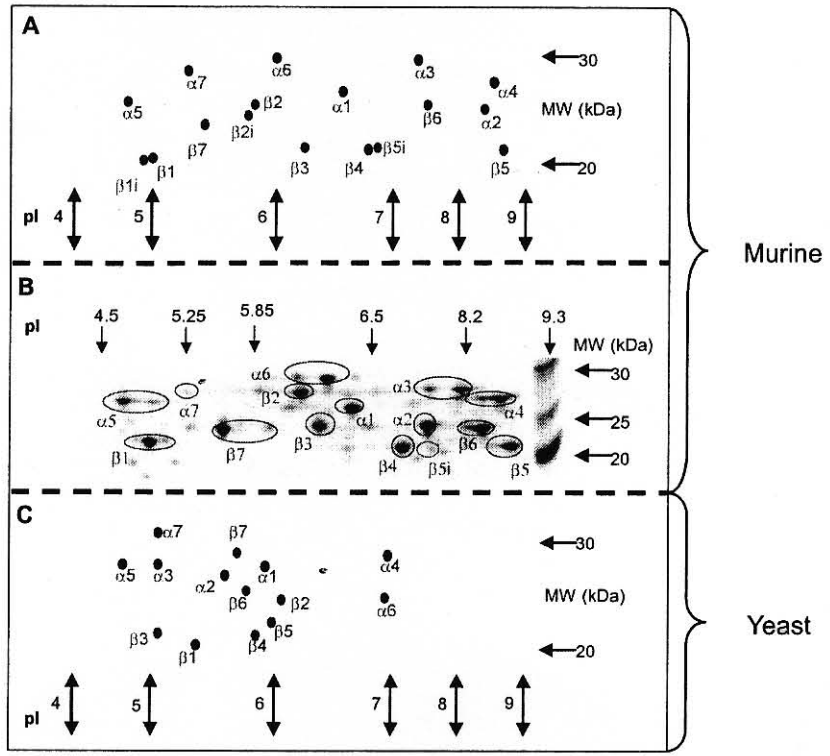


Table 1. Murine cardiac 20S proteasome subunits identified by LC/MS/MS. All 20S proteasome subunits were identified from murine heart, including constitutive subunits and inducible subunits. Theoretical pI of murine 20S proteasome is 6.0 (www.expasy.org), however, the experimental pI of murine cardiac 20S proteasome was 5.2. This indicated that 20S proteasome is post-translational modified in murine heart.

Gene Names	Common Names	# a.a	Theoretical M W	Estimated 2DE MW	Sequence Coverage
PSMA1	$\alpha 6$	263	29546	29.5 kDa	88%
PSMA2	$\alpha 2$	233	25794	23 kDa	77%
PSMA3	$\alpha 7$	254	28274	26.5 kDa	78%
PSMA4	$\alpha 3$	261	29471	28 kDa	91%
PSMA5	$\alpha 5$	241	26411	24.2 kDa	77%
PSMA6	$\alpha 1$	246	27372	25.4 kDa	63%
PSMA7	$\alpha 4$	248	27855	25.4 kDa	83%
PSMB1	$\beta 6$	240	26372	25.4 kDa	88%
PSMB2	$\beta 4$	201	22906	20 kDa	83%
PSMB3	$\beta 3$	205	22965	21 kDa	66%
PSMB4	$\beta 7$	219	24362	21 kDa	63%
PSMB5	$\beta 5$	205	22529	20 kDa	66%
PSMB6	$\beta 1$	205	21997	20 kDa	59%
PSMB7	$\beta 2$	234	25252	25.4 kDa	67%
PSMB8		204	22651	20 kDa	64%
PSMB9		199	21326	20 kDa	58%
PSMB10		234	24804	23 kDa	53%

20S Proteasome PI

Theoretical:
6.0
Experimental:
5.2

Figure 7. Electro-microscopic Analysis of Murine Cardiac 20S Proteasome. Murine cardiac 20S proteasome was imaged with Electro-microscopy after negative staining with uranic acid. The images were taken at 100,000X magnitude.

Electro-microscopic Image of 20S Proteasome (100,000X)

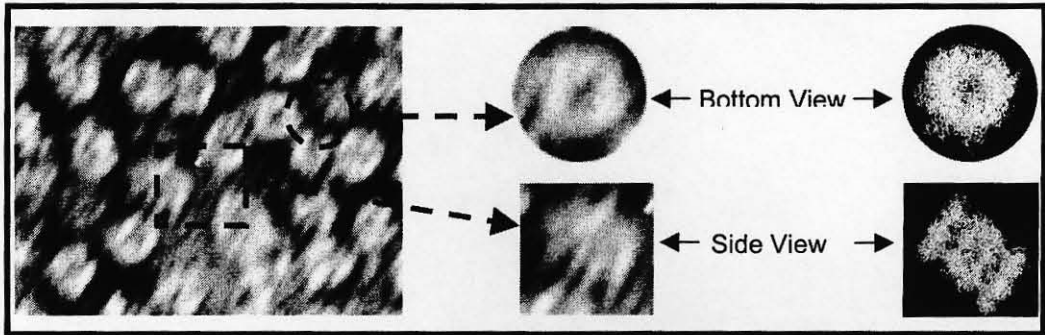


Figure 8. 2D Electrophoresis and Western-blotting Reveals Post-translation Modifications

on Multiple 20S Proteasome Subunits. 25ug of purified Murine cardiac 20S proteasome each was resolved by 2D electrophoresis (Bio-Rad), a total of 4 gels were run in parallel. Three of which was then transferred onto nitrocellulose membrane and immunoblotted with antibodies recognizing the phospho-Threonine, phospho-Serine, or phospho-Tyrosine proteins. Signal to noise ratio was set to be 2.5 folds higher than that of the background. The fourth 2D gel was stained with Sypro Ruby. The signals of phospho-proteasome subunits were identified by comparing/overlaying the 2D image of Sypro Ruby with that of the phospho-antibody gel.

Panel A.

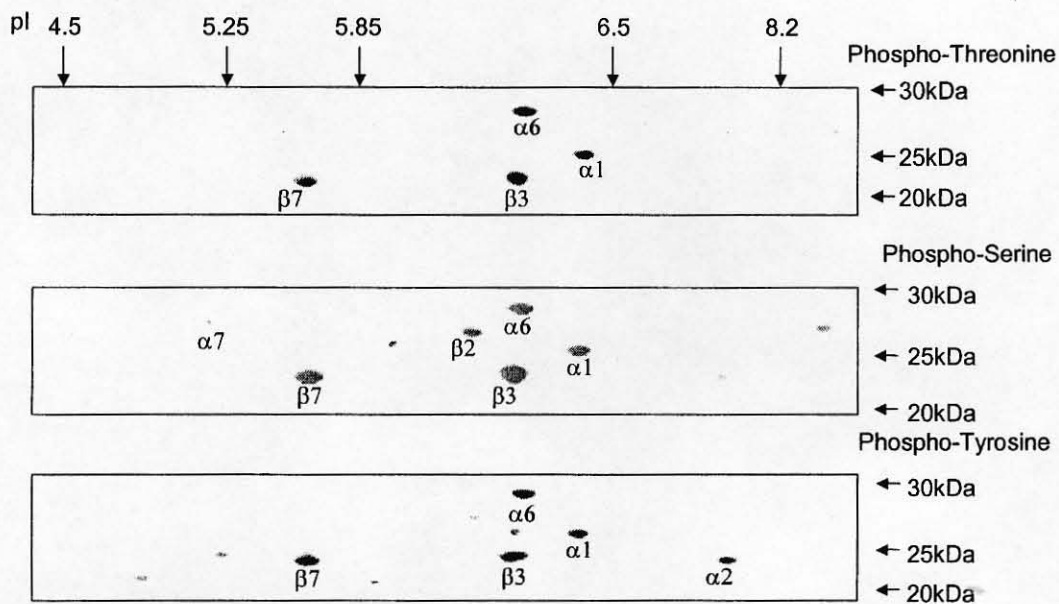
Upper membrane: Monoclonal antibody against phospho-threonine was purchased from Santa Cruz, which recognized subunits (alpha 1, 6 and beta 3,7)

Middle membrane: Polyclonal antibody against phospho-ser was purchased from Zymed, which recognized subunits (alpha 1,6,7 and beta 2,3,7). Although alpha7 signal was below our pre-set threshold, it has been confirmed with LC/MS/MS:

Lower membrane: Monoclonal antibody against phospho-tyr was purchased from Santa Cruz, which recognized a 1,2,6 subunits, and b 3, 7 subunits.

Panel B. Endogenous phosphorylation site of 20S proteasome α 7 subunit was identified with mass spectrometry to be Serine 249 residue at the C-terminal.

Panel A.



Panel B.

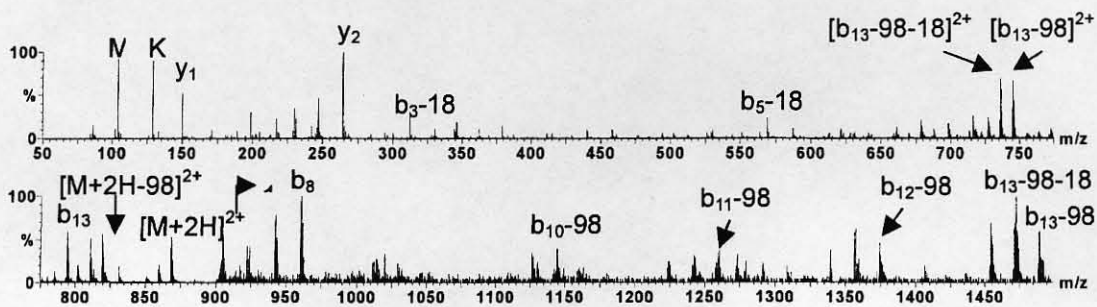


Figure 9. Protein Phosphatase 2A (PP2A) Associates with Murine Cardiac 20S Proteasome.

Purified murine cardiac 20S proteasome were resolve in SDS-PAGE in duplicate. **Panel A.** LC-MS/MS analysis of proteins resolved in one replica identified all three subunits of PP2A. The mascot report for subunit A was shown. **Panel B.** Western blot analysis of the other replica confirmed the presence of PP2A subunit C.

Panel A.

Match to: IPI00310091 Score: 1330

Tax_id=10090 Ensembl_locations(Chr-bp):17-19454629 Protein phosphatase 2, Subunit A

Nominal mass (M_r): 66119; Calculated pI value: 5.00

NCBI BLAST search of **PP2A_C** against nr

Fixed modifications: Carbamidomethyl (C)

Variable modifications: N-Acetyl (Protein), Oxidation (M), Pyro-glu (N-term E), Pyro-glu (N-term Q)

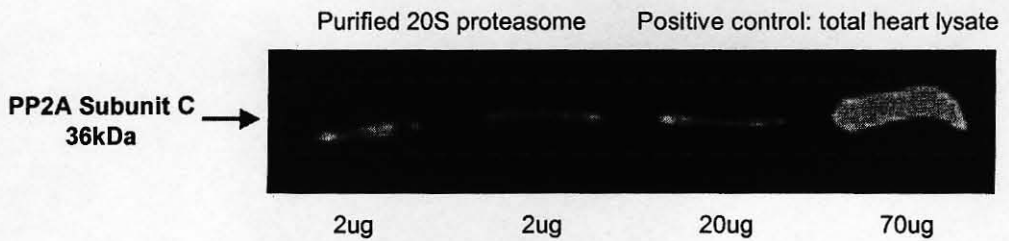
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: 45%

Matched peptides shown in **Bold Red**

```
1 MAAADGDDSL YPFAVLIDEL RNEDVQLRLN SIKKLSTIAL ALGVERTRSE
51 LLPFLTDTIY DEDEVLLALA EQLGTFITLV GGPEYVHCLL PPLESLATVE
101 ETVVRDKAVE SLRAISHEHS PSDLEAHFVP LVKRLAGGDW FTSRTSACGL
151 FSVCYPRVSS AVKAELRQYF RNLCSDDTPM VRRAAASKLG EFAKVLELDN
201 VKSEIIPMFS NLASDEQDSV RLLAVEACVN IAQLLPQEDL EALVMPTLRQ
251 AAEDKSWRVR YMVADKFTL QKAVGPEITK TDLVPAPQNL MKDCEAEVRA
301 AASHKVKEFC ENLSADCREN VIMTQILPCI KELVSDANQH VKSALASVIM
351 GLSPILGKDN TIEHLXPLFL AQLKDECEPV RLNIISNLDC VNEVIGIRQL
401 SQSLLPATIVE LAEDAKWRVR LAII EYMP LL AGQLGVEFFD EKLNSLCMAW
451 LVDHVYAIRE AATSNLKKLV EKFGKEWAHA TIIPKVLAMS GDPNYLHRMT
501 TLFCINVLSE VCGQDITTKH MLPTVLRMAG DPVANVRFNV AKSLQKIGPI
551 LDNSTLQSEV KPILEKLTQD QDVDVKYFAQ EALTVLSLA
```

Panel B.



**Table 2. 1D Electrophoresis and LC-MS/MS Analysis Identifies Critical 20S Proteasome
Associating Partners.**

Protein	Mascot Score	Mass (kDa)	Coverage (%)	Peptides (Unique/total)	Function	Previous Reports
Phosphatase PP2A, α subunit	1333	66023.1	44.8	26/35	Phosphatase Subunit.	Interact with Proteasome 20S subunits in yeast by Y2H (Nature 415:141-7).
PP2A, Subunit Catalytic	742	36155.6	56.3	15/21	Phosphatase Subunit.	
PP2A, Subunit Regulatory Kinase	89	52172.7	5.2	2/3	Phosphatase Subunit.	
PKA, α subunit, Catalytic	69	25268.1	13	2/2	Kinase	Arch Biochem Biophys 283:68-74
Casein Kinase II, β subunit	69	25268.1	13	2/2	Kinase	JBC 268:17413-7

CHAPTER III

PHOSPHORYLATION AS A KEY REGULATORY MECHANISM OF

THE CARDIAC 20S PROTEASOME

Introduction

The 20S proteasome is a stable protein complex with long half-life. On the other hand, the 20S proteasome assembly process is also prolonged for an enzyme. The dynamic regulation of 20S proteasome activities has to be achieved post-translationally for rapid responses, especially under stress conditions. Several forms of post-translational modifications (PTMs) occur on the proteasome. They could occur simultaneously or alternatively, including replacement of constitutive 20S proteasome subunits with inducible counterparts; selective association with activating/inhibiting protein complexes; and covalent PTMs (Coux, Tanaka et al. 1996; Rivett, Mason et al. 1997; Baumeister, Walz et al. 1998; Bochtler, Ditzel et al. 1999; Glickman and Ciechanover 2002).

Phosphorylation is the covalent post-translation addition of a phosphate group that has been drawing the most attention from biologists. The ubiquity of this modification and instant, reversible nature of this modification, justify this bias. Protein kinases transfer the γ -phosphate group of an ATP to certain amino acid residues of a protein substrate. Such modification mostly occurs at serine, threonine and tyrosine residues. Phosphorylations on histidine and aspartate residues also

occur, but are rare. Hundreds of kinases have been identified so far. They have great sequence diversity, but all share two homogenous domains for ATP binding and catalyzing phosphate group transfer, respectively. The remaining part of the kinase sequence dictates its binding affinity with other proteins and substrate preference. Consequently, a kinase can only phosphorylate the consensus sequence in certain substrates. The chemical property of hydroxyl groups is similar between serine and threonine residues, which are distinct from that of tyrosine. Accordingly, kinases are catalogued into two families: serine/threonine kinases preferably phosphorylate serine and threonine residues and tyrosine kinases preferably phosphorylate tyrosine residues of a protein. The specificity of the kinase enables it to play an important role in certain signaling cascades. Protein phosphatases are a group of enzymes that counteract the function of kinases by removing a phosphate group from phosphorylated-protein substrates. Serine/threonine phosphatases, tyrosine phosphatases, alkaline phosphatases and acid phosphatases constitute four families of phosphatases. Compared to kinases; the substrate bias is much lower for phosphatases. Correspondingly, there are fewer protein phosphatases than kinases within the cells. The phosphorylation status of a protein affects its enzymatic activity, subcellular localization, and half-life. Kinases and phosphatases coordinately control intracellular phosphorylation signaling cascades, which is important for the cell to maintain homeostasis and response to environmental changes (Johnson and Lewis 2001).

Phosphorylations have been identified in the 20S proteasome purified from both yeast and a few mammalian tissues, such as kidney, liver, etc. The phosphorylation profile of the 20S proteasome from the murine heart is lacking (Pereira and Wilk 1990; Castano, Mahillo et al. 1996; Bose, Mason et al. 1999; Feng, Longo et al. 2001; Fernandez Murray, Pardo et al. 2002; Iwafune, Kawasaki et al. 2002; Bose, Stratford et al. 2004). According to the studies described in

previously section, the 20S proteasome was endogenously phosphorylated in the murine heart. The study presented in this chapter further confirmed the murine cardiac 20S proteasome was phosphorylated. Moreover, attempts have been made to put these phosphorylations into a biological context.

In certain mammalian tissues, casein kinase II (Ck2), PKA and polo-like kinases have been reported as 20S proteasome associating kinases, which could directly control the 20S proteasome phosphorylation state (Pereira and Wilk 1990; Castano, Mahillo et al. 1996; Marambaud, Wilk et al. 1996; Feng, Longo et al. 2001). However, there is no such study for mammalian cardiac tissue. PKA and polo-like kinases have been reported capable of increasing 20S proteasome $\beta 5$ subunits activity. The effect of CK2 phosphorylation on the 20S proteasome function has been contradicting in different reports. In part II of this thesis, CK2 and PKA have been identified as 20S proteasome associating kinases in murine heart, which is consistent with other tissues. Protein phosphatase 2A (PP2A) has been identified as the 20S proteasome associating phosphatase in murine heart. A yeast-two-hybrid study suggested PP2A might interact with 20S proteasome. There is no previous proteomic study reported PP2A interacting with 20S proteasome in mammalian tissues. A comprehensive 20S proteasome functional study is needed to uncover how murine cardiac 20S proteasome are regulated functionally by these kinases/phosphatase.

Materials and Methods

Purified Murine Heart 20S Proteasome

10g of mice heart tissue was homogenized by a potter homogenizer in homogenize buffer (Tris 20mM pH 7.8, EDTA 0.1mM, DTT 1mM, with protease inhibitor cocktail from Roche and phosphatase cocktails from Sigma). Cytosolic fraction was collected as the supernatant after 2hr centrifugation at 25,000g. Cytosolic fraction was then fractionated by ammonium sulfate precipitation. The pellet collected between 40% to 60% ammonium sulfate saturation was re-suspended in 10ml dialysis buffer (Tris 20mM pH 7.4, MgCl₂ 5mM, DTT 0.5mM). The re-suspended fraction was dialyzed again with 4L dialysis buffer overnight, to remove ammonium sulfate from it. The dialysate was fractionated with preparative-scale strong-anion-ion-exchange column (Q FastFlow resin packed in XK 26/40 column from GE healthcare) by stepwise salt concentration gradient elution at flow rate 5ml/min (45% B, until UV280nm monitor reading goes to baseline; 75% B, collect 200ml; 100% B, until UV280nm monitor goes to baseline. Buffer A: Tris 20mM pH 7.4, MgCl₂ 5mM, DTT 0.5mM, Glycerol 10%; Buffer B: Tris 20mM pH 7.4, MgCl₂ 5mM, DTT 0.5mM, Glycerol 10%, KCl 600mM). 75% B fraction enriched in 20S proteasome. This fraction was centrifuged at 5°C 42,000rpm (Ti45 fixed angle rotor from Beckman) for 19hr. The pellet was collected, re-suspended in buffer A and resolved with analytical-scale strong-ion-exchange column (Mono Q HR5/50 from GE healthcare) with a linear salt concentration gradient from 0% B to 100% B through 17.5 column volume. The purified 20S proteasome was recovered in fractions around 60%B.

Calf Intestinal Alkaline Phosphatase

Calf Intestinal Alkaline Phosphatase (CIAP) was purchased from Promega; Agarose crosslinked CIAP was purchased from Sigma. 10X CIAP assay buffer (0.5M Tris-HCl pH 8.5, 10mM MgCl₂, 1mM ZnCl₂) was prepared using reagents obtained from Sigma.

Phosphatase Inhibitors

The protein phosphatase 2A specific inhibitor, okadaic acid, was purchased from Calbiochem. Okadaic acid does not affect tyrosine phosphatase, acid phosphatase and alkaline phosphatase. Its specificity towards protein phosphatase 2A is 100 folds higher than that towards protein phosphatase 1 (PP1).

Kinase Inhibitors

PKA specific inhibitor, H-89, was purchased from Calbiochem.

CK2 specific inhibitor, 4,5,6,7-Tetrabromobenzotriazole, was purchased from Calbiochem.

CIAP Treatment of 20S Proteasome

CIAP treatment of 20S proteasome: To different wells of a 96-well microplate, 0.065 μ g purified murine cardiac 20S proteasome incubated with CIAP at 37°C. Then 10X 20S proteasome assay buffer and 10X 20S proteasome substrate stock were added immediately or after 30min incubation. After 1hr incubation at 37°C, the proteasome activity was measured using fluorescently labeled substrates.

CIAP-agarose treatment of 20S proteasome: To each micro-centrifuge tube, 0.065 μ g purified murine cardiac 20S proteasome incubated with CIAP at 37°C for 30min. CIAP-agarose was removed by centrifugation; supernatant was transferred to 96-well microplate. Then 10X 20S proteasome assay buffer and 10X 20S proteasome substrate stock were added. After 1hr incubation at 37°C, proteasome activity was measured using fluorescently labeled substrates.

Phosphorylation of 20S Proteasome

20S proteasome was incubated with PKA (Sigma) in assay buffer (50mM Tris-HCl, pH 7.5, 20mM MgCl₂, 1mM DTT, 2mM ATP) at 35°C for 15min. Then the reaction mixture was dispensed into 96-well microplate, complemented with 10X assay buffer and 10X substrate stock. After 1hr

incubation at 37°C, the proteasome activity was measured using fluorescently labeled substrates.

20S proteasome was incubated with casein kinase II (New England Biolabs) in assay buffer (50mM Tris-HCl, pH 7.5, 20mM MgCl₂, 1mM DTT, 2mM ATP) at 35°C for 15min. Then the reaction mixture was dispensed into 96-well microplate, added with 10X assay buffer and 10X substrate stock. After 1hr incubate at 37°C, proteasome activity was measured using fluorescently labeled substrates.

Fluorescent Peptide Substrates and 20S Proteasome Activities Assay

Z-Leu-Leu-Glu-AMC from Sigma was used to measure β 1 subunit activity. (10X assay buffer: 250mM HEPES, pH 7.5; 5mM EDTA; 0.5% NP-40; 0.01% SDS). Boc-Leu-Ser-Thr-Arg-AMC from Sigma was used to measure β 2 subunit activity. (10X assay buffer: 250mM HEPES, pH 7.5; 5mM EDTA; 0.5% NP-40; 0.01% SDS). Suc-Leu-Leu-Val-Tyr-AMC from Bachem was used to measure β 5 subunit activity. (10X assay buffer: 250mM HEPES, pH 7.5; 5mM EDTA; 0.3% SDS).

Different concentrations of substrate (0, 2, 10, 20, 50, 100, 200, 500 μ M) were used in 20S proteasome activity assays to generate substrate concentration dependent activity curves.

SDS-PAGE and Phosphor-protein Detection by Pro-Q Staining

Purified murine cardiac 20S proteasome incubated with CIAP-agarose or albumin-agarose resin at 37°C for 30min. Then the beads were removed by centrifugation. Supernatant were collected and resolved by SDS-PAGE. The proteins in the gel was fixed and stained with phospho-protein specific dye according to standard protocol from the vendor (Pro Q Diamond from Molecular Probes).

Statistical Analysis

All data are presented as Mean \pm S.E. Groups are compared using the student's *t* test for unpaired data. A *P* value of less than 0.05 was considered significant.

Results

CIAP Treatment Removes Phosphate Groups from Phosphorylated 20S Proteasome

Purified murine cardiac 20S proteasome were treated with CIAP (buffer only treatment serves as negative control). Then the 20S proteasome was resolved with SDS-PAGE and stained with a phosphor-protein specific dye (Pro-Q Diamond from Molecular Probes) and total protein stain (SYPRO Ruby from Molecular Probes) sequentially. The fluorescent signal of SYPRO Ruby stain showed no intensity differences between the treated and negative control (Figure 10, Panel B). However, between the same two groups, Pro-Q Diamond staining resulted in distinct fluorescent signals (Figure10, Panel A). The murine cardiac 20S proteasome was phosphorylated endogenously and these phosphate groups could be removed by CIAP treatment.

CIAP Treatment Selectively Increases 20S Proteasome Peptidase Activities

CIAP treatment also regulates 20S proteasome peptidase activities. Two CIAP treatment protocols were used. According to the first protocol, 20S proteasome and CIAP were added to the microplate simultaneously with 20S proteasome assay buffer and 20S proteasome substrate, which was set as time 0. Fluorescent signals from cleaved substrates were recorded in an hour at 37°C to measure peptidase activities. The result showed β 1 subunit activity did not change significantly. β 2 subunit activity increased significantly, while β 5 subunit activity was slightly inhibited (Figure 11, Panel A).

According to the second protocol, the 20S proteasome was pre-treated with CIAP for 30min at 37°C. At time 0, proteasome assay buffer and substrate were added to evaluate proteasome

activities within an hour at 37°C. In this setup, β 1 subunit activity remained unchanged. β 2 subunit activity was enhanced dramatically. Notably, the β 5 subunit activity was upregulated in this condition (Figure 11, Panel B).

Removal of CIAP from the De-phosphorylated 20S Proteasome

Increases the Peptidase Activities Greater than When CIAP is Present

Agarose-crosslinked CIAP was obtained from Sigma. This form of CIAP could be removed after treatment with a simple centrifugation procedure. This enabled us to isolate the 20S proteasome from the CIAP.

Purified murine cardiac 20S proteasome was pre-treated with CIAP-agarose beads for 30min at 37°C. At time 0, CIAP-agarose beads were removed; proteasome assay buffer and substrate were added to evaluate proteasome activities within an hour at 37°C. β 1 subunit activity showed no significant difference. β 2 subunit activity was enhanced significantly. Importantly, in this condition, β 5 subunit activity was enhanced the most significantly (Figure 12, Panel A).

In another assay, purified murine cardiac 20S proteasome was pre-treated with CIAP-agarose for 30min at 37°C. At time 0, CIAP-agarose beads were removed; proteasome assay buffer and substrate were added along with recombinant CIAP. After 1hr incubation at 37°C, 20S proteasome activities were measured via recording fluorescent emission. β 1 subunits showed no significant difference. β 2 subunit activity was induced significantly. Importantly, β 5 subunit activity was enhanced less significantly than previous setup, which mimicked the results of recombinant CIAP pre-incubation (Figure 12, Panel B).

Casein Kinase II Co-purifies with 20S Proteasome

Purified murine cardiac 20S proteasome were pre-incubated with various amount of CKII (Trace amount of CKII had been showed to co-purify with murine cardiac 20S proteasome) at 35°C for 15min in phosphorylation buffer (50mM Tris-HCl, pH 7.5, 20mM MgCl₂, 1mM DTT, 2mM ATP). At time 0, proteasome assay buffer and substrate were added to evaluate proteasome activities within an hour at 37°C. Data showed all three proteasome peptidase activities were enhanced by this treatment (Figure 13).

Phosphorylation of 20S Proteasome Changes 20S Proteasome Activities

Purified murine cardiac 20S proteasome were pre-incubated with various amount of PKA (Trace amount of PKA had been showed to co-purify with murine cardiac 20S proteasome) at 35°C for 15min in phosphorylation buffer (50mM Tris-HCl, pH 7.5, 20mM MgCl₂, 1mM DTT, 2mM ATP). At time 0, the proteasome assay buffer and substrate were added to evaluate proteasome activities within an hour at 37°C. Data showed all three proteasome peptidase activities were also enhanced by this treatment (Figure 14).

Protein Phosphatase 2A Co-purifies with 20S Proteasome, Which may Regulate 20S

Proteasome Activities by Removing Phosphate Groups from the 20S Proteasome *in vivo*

Purified murine cardiac 20S proteasome were pre-incubated with various amount of PP2A inhibitor, okadaic acid (PP2A had been showed to co-purify with murine cardiac 20S proteasome)

at 35°C for 30min. At time 0, proteasome assay buffer and substrate were added to evaluate proteasome activities within an hour at 37°C. Treatment of purified murine cardiac 20S proteasome with PP2A inhibitor significantly increased all β subunit activities, especially β 1 and β 5 (Figure 15, Panel A).

Purified murine cardiac 20S proteasome were pre-incubated with various amount of PP2A at 35°C for 15min. At time 0, proteasome assay buffer and substrate were added to evaluate proteasome activities within an hour at 37°C. Treatment of purified murine cardiac 20S proteasome with PP2A significantly decreased β 1 and β 5 subunit activities (Figure 15, Panel B).

Discussion

20S Proteasome is Phosphorylated *in vivo*

20S proteasome has been shown to be phosphorylated endogenously in yeast, rice and several mammalian tissues. Several subunits are phosphorylated in a similar fashion in all these samples, while there are also subunits showing clear distinction on phosphorylation status among samples.

As described in detail in part II, the murine cardiac 20S proteasome was purified and western blotting with phosphor-protein specific antibodies and mass spectrometric showed that this cardiac 20S proteasome was phosphorylated endogenously. In a CIAP de-phosphorylation/Pro-Q phosphor-protein stain study; this was further confirmed. Phosphorylation is one of the important mechanisms to regulate 20S proteasome in murine heart.

The Choice of Detergents has an Effect on 20S Proteasome Activities Assay

The 20S proteasome is a latent protein complex with peptidase activities. The two seven-subunits α rings impose this latency. In latent state, N-terminal sequences of α subunits block the entrance for substrates to reach the catalytic core. *In vivo*, this latency can be relieved by activator protein complexes, including 19S regulatory particles, 11S and PA200, which can open the entrance by inducing conformation changes of these N-terminal sequences. *In vitro*, this activation can be mimicked with detergents, which can also induce conformational changes. Out of the various choices of detergents, SDS is most commonly used, even though, there are reports that show that SDS is not optimal for all subunit activity assays. The popularity of SDS has both a historical reason and an economical reason. During the early days of proteasome activity studies, the $\beta 5$ subunit activity assay was used as single criteria to evaluate the function of this protein

complex, and SDS was optimal for this assay. As more and more researchers realize the needs of comprehensive evaluation of 20S proteasome activities by measuring all three 20S proteasome peptidase activities, it required the establishment of optimal assay protocols for each peptidase activity assay. NP-40 is another detergent that has been used in proteasome activity assay. The efficiency of SDS and NP-40 in inducing the 20S proteasome peptidase activities was compared. Data showed NP-40 was a better detergent for $\beta 1$ and $\beta 2$ subunit activity assays, while SDS was the better detergent for the $\beta 5$ subunit activity assay.

CIAP Treatment Removes the Phosphorylation-dependent Inhibition of 20S Proteasome

CIAP treatment removed serine, threonine phosphorylation and tyrosine phosphorylation. As to its name, Calf Intestinal Alkaline phosphatase has optimal enzymatic activity at alkaline pH, which is 9.3. However, 20S proteasome is less stable beyond pH 8.5. Excess molar amount of CIAP has to be used to de-phosphorylate 20S proteasome completely. Pro-Q diamond is a fluorescent dye that selectively recognizes phospho-proteins. Pro-Q diamond staining showed CIAP treatment could efficiently remove phosphate groups from 20S proteasome.

Comparing 20S proteasome activities with or without CIAP complete de-phosphorylation, it showed phosphate conjugation having significant functional significance. $\beta 1$ subunit activity was not altered by complete de-phosphorylation with CIAP. The $\beta 2$ activity was significantly enhanced while the effect on the $\beta 5$ subunit activity was relative complicated. The $\beta 5$ activity was slightly decreased if activity assay was conducted simultaneously with CIAP treatment, while its activity was significantly increased if proteasome activity assay was conducted after 30min CIAP pre-treatment. This raised the possibility that CIAP can be degraded by 20S proteasome. As a substrate, CIAP competes proteolytic active sites with peptide substrates, which would decrease 20S proteasome activity measured with peptide substrates. In an inhibition study, the $\beta 5$ subunit

was reported as the main subunit responsible for protein degradation. Its inhibition resulted in 50% less in protein degradation rate. Several proteins have been reported to be degraded by 20S proteasome independent of ubiquitin. This could be the explanation for distinct effects with different treatment protocols. CIAP pre-incubation partially separated proteasome assay procedure from the CIAP treatment procedure.

Removal of CIAP after Treatment of the 20S Proteasome is Important to Characterize the Full Effects of De-phosphorylation on the 20S Proteasome Activities

The commercial availability of agarose resin crosslinked CIAP enabled us to completely separate the CIAP treatment procedure from the proteasome assay procedure. After treatment, this resin could be easily removed from a reaction mixture by a simple centrifugation procedure. The effect of CIAP resin treatment was consistent with previous data gained using recombinant CIAP. β 1 activity showed no change; β 2 and β 5 activities significantly induced. The dramatic increase in β 5 activity supported our hypothesis that recombinant CIAP could be a substrate of 20S proteasome and act as competitive inhibitor for β 5 subunit activity assay. The further test this hypothesis; A parallel experiment was conducted. The 20S proteasome was treated with CIAP-agarose resin. This time, after removal of the resin, recombinant CIAP was added back to the reaction mixture at time 0 of the 20S proteasome activity assay. As expected, in this parallel experiment, the β 5 activity increased at a significant lower magnitude compare to when the CIAP was removed. In short, complete de-phosphorylation of the 20S proteasome with excess CIAP significantly enhanced its trypsin-like (β 2) and chymotrypsin-like (β 5) peptidase activities, while caspase-like activity was not significantly affected. However, recombinant CIAP could be degraded by 20S proteasome and CIAP has the effect as a competitive inhibitor.of the β 5 activity.

Potential Kinases Which Regulates the 20S Proteasome Activities *in vitro* and *in vivo*

20S proteasome purified from murine heart was endogenously phosphorylated. This is consistent with the finding from yeast and other mammalian tissues. The identification of kinases and phosphatases involved is a critical step toward elucidating signaling cascades that regulate 20S proteasome activities. This would help us understand 20S proteasome functions in physiological and pathological conditions and design specific pharmacological agents.

The improvement of mass spectrometry technologies makes systemic characterization of 20S proteasome associating kinases and phosphatases possible. The state-of-the-art mass spectrometers have the ability to detect proteins at higher sensitivity than most protein dyes commercially available. Taking advantages of the advance in technology, we identified CKII, PKA and PP2A as 20S proteasome associating proteins. The identification of CK2 and PKA were consistent with reports from other tissues and species. In a high-throughput yeast-two-hybrid study, PP2A is suggested as a potential 20S proteasome interacting protein, while there is no previous direct report on PP2A as endogenous 20S proteasome associating protein. In this study, mass spectrometry and western blot confirmed that PP2A was one of the 20S proteasome associating proteins.

Kinases and Phosphatases Coordinately Regulate the Activities of 20S Proteasome *in vivo*

As mentioned in previous section, CKII, PKA and PP2A were identified as 20S proteasome associating proteins in murine heart. The regulation of 20S proteasome by these enzymes is an important part of its biochemistry. PKA, CKII and PP2A all have been reported to have important roles in cardiac physiology. PKA is a component of β -adrenergic receptor cascade. CKII participates in apoptotic pathway. PP2A has been reported to be involved in hypertrophy.

Both CKII and PKA phosphorylations increased 20S proteasome activities while PP2A

showed the opposite effect. Both PKA and CKII belong to serine/threonine kinase family. PP2A is one of the few serine/threonine phosphatases. Both PKA and CKII are serine/threonine kinases and PP2A is serine/threonine phosphatase. CIAP is a member of another family of phosphatase, alkaline phosphate. It is capable of removing serine, threonine and tyrosine phosphorylation in a less specific manner. The CIAP treatment effect suggested there are more kinases/phosphatases involved in 20S proteasome regulation. These enzymes were not identified in the purified 20S proteasome preparation from Murine heart, probably due to less affinity and/or lower stoichiometry to the 20S proteasome.

Summary

20S proteasome has been proved to be phosphorylated in the murine heart by multiple methods, including proteomic method, western blot and biochemical method. As a stable protein complex with long half-life, phosphorylation modifications are necessary to accommodate this mega-protease to signaling cascades.

Being the final and only executor of ubiquitin-proteasome system, 20S proteasome is controlled by different signaling pathways. The complexity of murine cardiac 20S proteasome phosphorylation profiles reflects this coordinated control scenario. Proteomic part of this study has identified several kinases and phosphatase as 20S proteasome interacting proteins, including CKII, PKA and PP2A, which are candidates controlling its phosphorylation profile and activities in the murine heart. CKII and PKA have been reported as 20S proteasome associating proteins in other tissue, but not in the heart. The studies of their impact on 20S proteasome activities are incomplete and even controversial in the case of CkII. PP2A has just been suggested in a yeast-two-hybrid experiment as 20S proteasome associating protein. The functional impact of this association has not been demonstrated at the tissue level before.

In this study, two approaches have been taken to study the regulatory roles of phosphorylation on murine cardiac 20S proteasome. One approach was to globally de-phosphorylated 20S proteasome with CIAP and observe its outcome. Further studies were conducted to study the effect of particular kinases or phosphatase.

CIAP is a phosphatase with limited specificity. Its treatment has been proved to remove phosphate groups from 20S proteasome. There are two intriguing observations. This treatment significantly enhanced 20S proteasome β 2 subunit activity. Using different treatment protocols, β 5

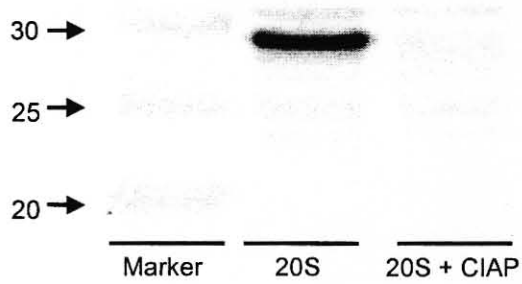
subunit activity was affected differentially. With the availability of agarose-linked CIAP, the de-phosphorylation step and 20S proteasome activity assay step could be separated. This documented global de-phosphorylation with CIAP increased both β 2 and β 5 subunits activities and CIAP were degraded by 20S proteasome.

Compared to global de-phosphorylation study, studies with specific kinases and phosphatase are of more biological significance. PKA and CKII both are serine/threonine kinases. PKA is part of β -adrenergic receptor signaling cascade; CKII involves cell survival signaling. PP2A is a serine/threonine phosphatase, and it has been suggested as a tumor suppressor. PKA and CKII treatment both enhanced the peptidase activities of 20S proteasome. On the contrary, inhibition of 20S proteasome co-purifying PP2A also enhanced its peptidase activities. These results uncovered the control of 20S proteasome and put it in the context of phosphorylation cascades, which is important to study its role in cardiac physiology.

Figure 10. CIAP Treatment Removes Phosphate Groups from Phosphorylated 20S Proteasome. Purified murine cardiac 20S proteasome were treated with CIAP for 30min at 37°C and then resolved with SDS-PAGE. **Panel A.** The gel was stained with phospho-protein specific dye (Pro-Q Diamond from Molecular Probes). Compare to the non-CIAP treated 20S proteasome, the 20S proteasome was de-phosphorylated by this treatment. **Panel B.** After Pro-Q Diamond staining, the fluorescence from the gel was bleached with light and then the same gel was stained with SYPRO Ruby total protein stain. The SYPRO Ruby stain pattern was not significantly different between the CIAP treated and non-treated 20S proteasome.

Panel A.

MW (kDa) Pro-Q Phospho-protein Staining



Panel B.

MW (kDa) SYPRO Ruby Protein Staining

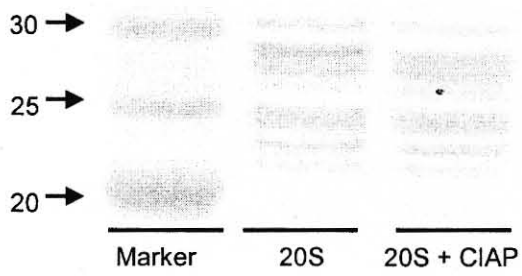
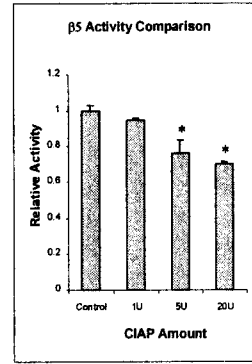
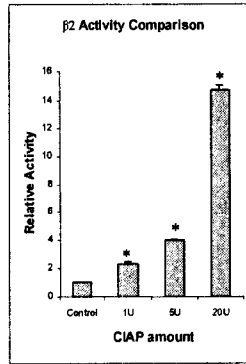
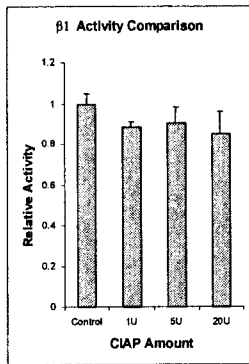


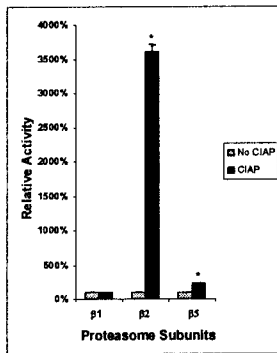
Figure 11. CIAP Treatment Selectively Increases 20S Proteasome Peptidase Activities.

Panel A. Purified murine cardiac 20S proteasome was co-incubated with both CIAP (Promega) and subunit specific 20S proteasome substrates (Z-Leu-Leu-Glu-AMC for $\beta 1$ subunit; Boc-Leu-Ser-Thr-Arg-AMC for $\beta 2$ subunit; Suc-Leu-Leu-Val-Tyr-AMC for $\beta 5$ subunit) at 37°C for 1hr. Proteasome activities were measured according to AMC fluorescence emission after it was released from the recombinant peptides. CIAP treatment in this manner induced no significant difference in $\beta 1$ subunit activity, while $\beta 2$ subunit activity was significantly enhanced and $\beta 5$ subunit activity inhibited. **Panel B.** Purified murine cardiac 20S proteasome was pre-incubated with CIAP (Promega) at 37°C for 30min. Then subunit specific 20S proteasome substrates (Z-Leu-Leu-Glu-AMC for $\beta 1$ subunit; Boc-Leu-Ser-Thr-Arg-AMC for $\beta 2$ subunit; Suc-Leu-Leu-Val-Tyr-AMC for $\beta 5$ subunit) were added and the mixture were incubated at 37°C for 1hr. Proteasome activities were measured according to AMC fluorescence emission released from the recombinant peptides. CIAP treatment in this manner induced no significant difference in $\beta 1$ subunit activity, while both $\beta 2$ and $\beta 5$ subunit activities were significantly enhanced.

Panel A.



Panel B.

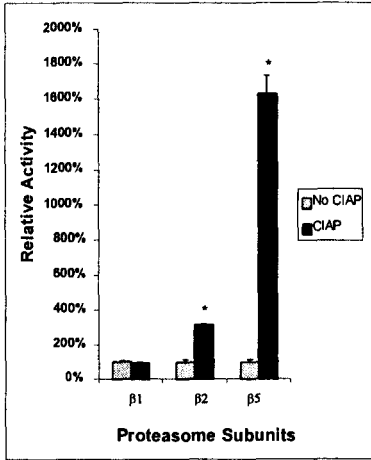


n=3
* p<0.05

n=6
* p<0.05

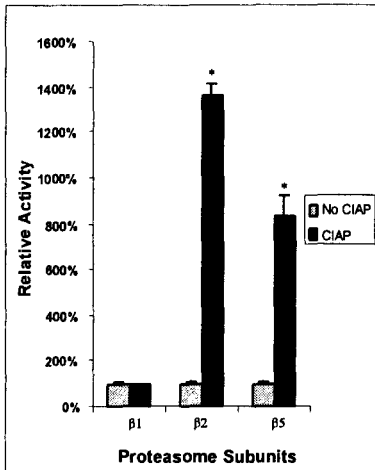
Figure 12. Removal of CIAP from the De-phosphorylated 20S Proteasome Increases the Peptidase Activities Greater than When CIAP is Present. Panel A. Purified murine cardiac 20S proteasome was pre-incubated with agarose-crosslinked CIAP (Sigma) at 37°C for 30min. CIAP agarose was removed and subunit specific 20S proteasome substrates (Z-Leu-Leu-Glu-AMC for β 1 subunit; Boc-Leu-Ser-Thr-Arg-AMC for β 2 subunit; Suc-Leu-Leu-Val-Tyr-AMC for β 5 subunit) were added. The mixtures were incubated at 37°C for 1hr. Proteasome activities were measured according to AMC fluorescence emission after it was released from the recombinant peptides. CIAP treatment in this manner induced no significant difference in β 1 subunit activity, while both β 2 and β 5 subunit activities were significantly enhanced. The increase in β 5 subunit activity was higher than that of β 2 activity. **Panel B.** Purified murine cardiac 20S proteasome was pre-incubated with agarose-crosslinked CIAP (Sigma) at 37°C for 30min. CIAP agarose was removed and subunit specific 20S proteasome substrates (Z-Leu-Leu-Glu-AMC for β 1 subunit; Boc-Leu-Ser-Thr-Arg-AMC for β 2 subunit; Suc-Leu-Leu-Val-Tyr-AMC for β 5 subunit) were added along with recombinant CIAP. The mixtures were incubated at 37°C for 1hr. Proteasome activities were measured according to AMC fluorescence emission after it was released from the recombinant peptides. CIAP treatment in this manner induced no significant difference in β 1 subunit activity, while both β 2 and β 5 subunit activities were significantly enhanced. The increase in β 2 subunit activity was higher than that of β 5 activity.

Panel A.



n=9
* p<0.05

Panel B.



n=3
* p<0.05

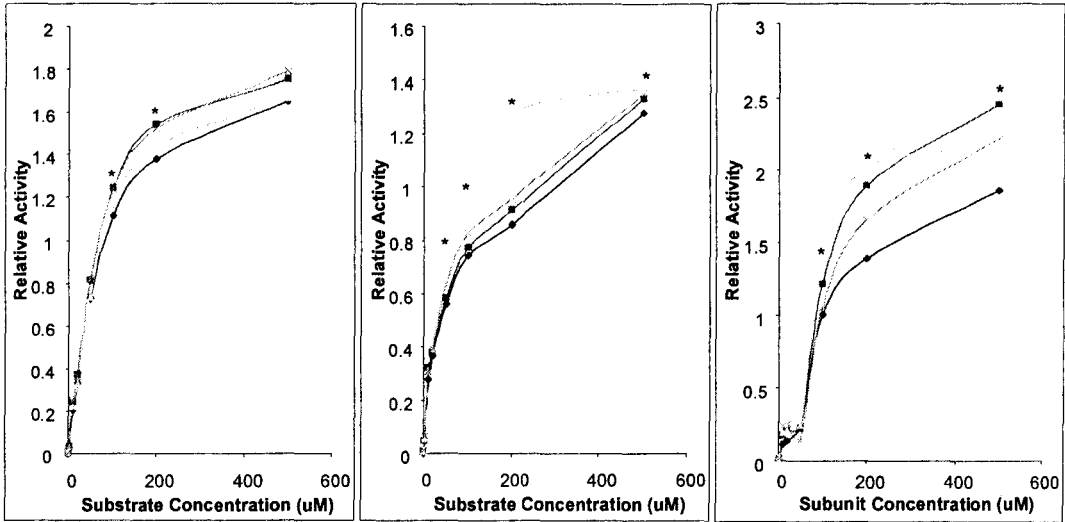
Figure 13. Casein Kinase II Co-purifies with 20S Proteasome and Regulates Its Activities.

Purified murine cardiac 20S proteasome was incubated at 35°C with CKII in phosphorylation buffer (50mM Tris-HCl, pH 7.5, 20mM MgCl₂, 1mM DTT, 2mM ATP) for 15min. Then proteasome peptidase activities were measured with fluorescently labeled recombinant peptide substrates. All three peptidase activities were enhanced after the incubation.

β 1 Subunit Activity Assay

β 2 Subunit Activity Assay

β 5 Subunit Activity Assay



n=3, * p<0.05

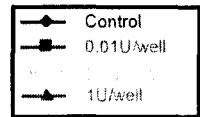
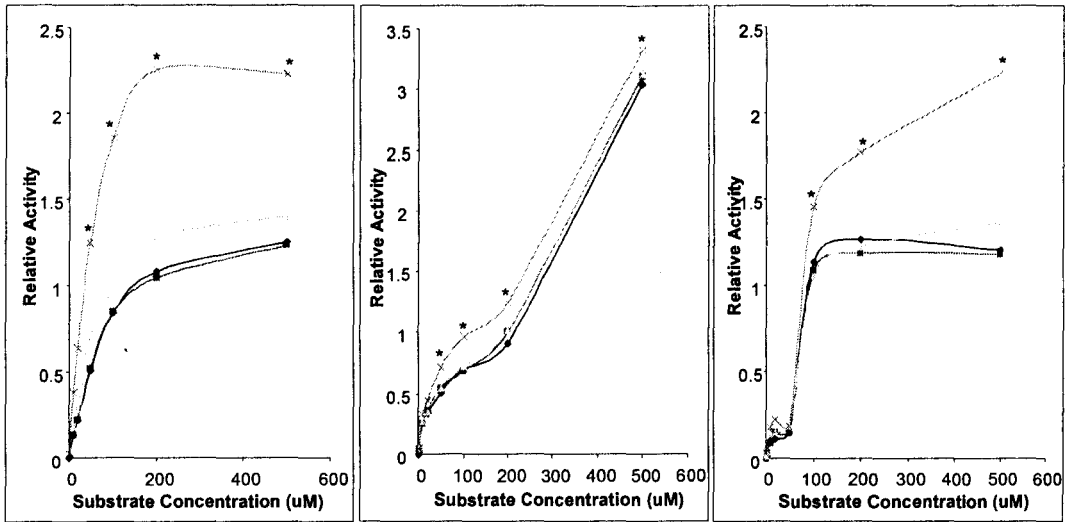


Figure 14. Phosphorylation of the 20S Proteasome by PKA Changes 20S Proteasome

Activities. Purified murine cardiac 20S proteasome was incubated at 35°C with PKA in phosphorylation buffer (50mM Tris-HCl, pH 7.5, 20mM MgCl₂, 1mM DTT, 2mM ATP) for 15min. Then the proteasome peptidase activities were measured with fluorescently-labeled recombinant peptide substrates. All three peptidase activities were enhanced after the incubation.

β 1 Subunit Activity Assay β 2 Subunit Activity Assay β 5 Subunit Activity Assay



n=3, * P<0.05

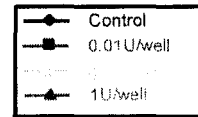
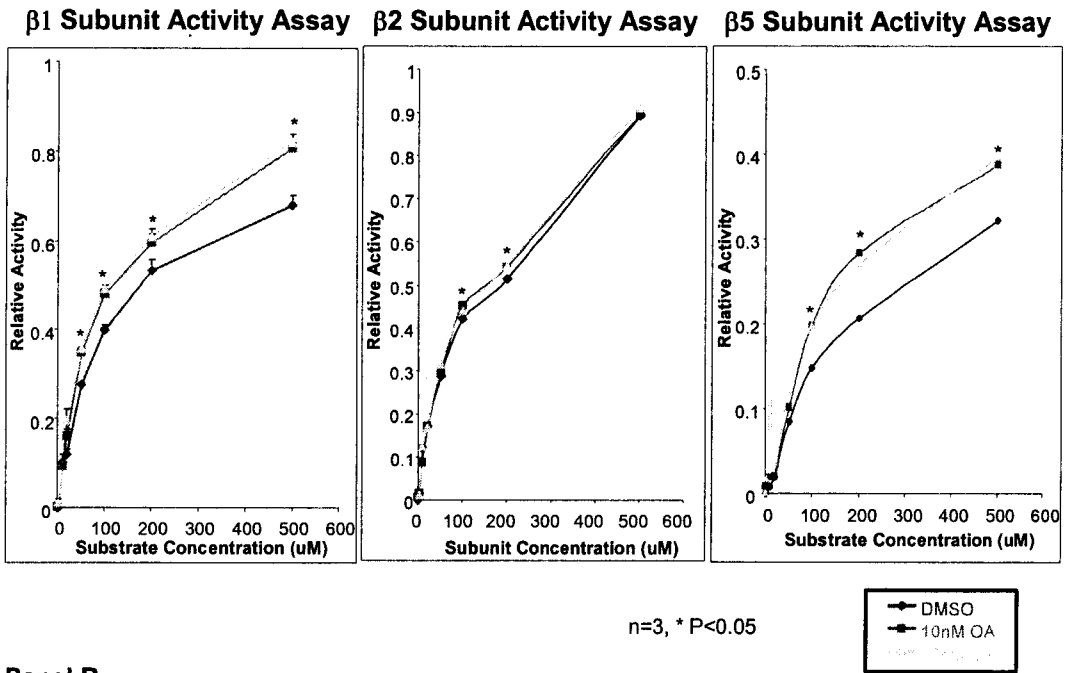
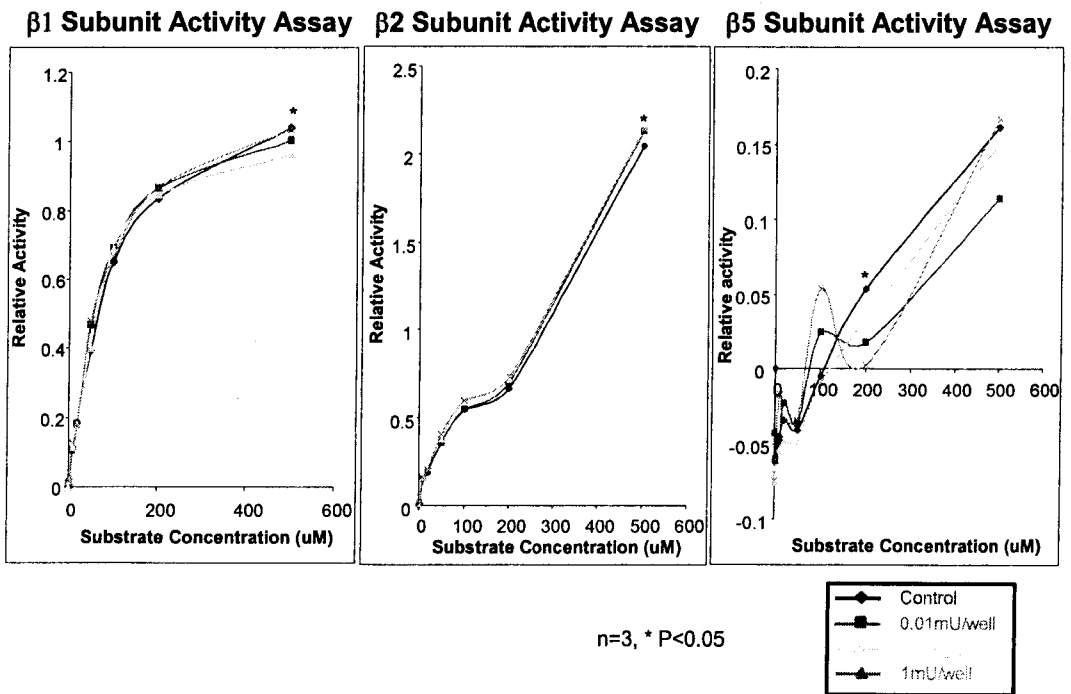


Figure 15. Inhibition of the Murine Cardiac 20S Proteasome Co-purifying Protein Phosphatase 2A Enhances Proteasome Peptidase Activities. Panel A. Purified murine cardiac 20S proteasome was incubated at 35°C with okadaic acid (inhibited 20S proteasome co-purifying PP2A) for 30min. Then proteasome peptidase activities were measured with recombinant peptide substrates. All three peptidase activities were enhanced after the incubation. **Panel B.** Purified murine cardiac 20S proteasome was incubated at 35°C with PP2A for 15min. Then proteasome peptidase activities were measured with recombinant peptide substrates. β 1 and β 5 subunit activities were decreased after incubation.

Panel A.



Panel B.



CHAPTER IV.

ASSOCIATION OF α B-CRYSTALLIN MODULATES 20S PROTEASOME ACTIVITIES

Introduction

In the cell, 20S proteasome interacts with various proteins. These interactions regulate both the activity and the subcellular localization of the 20S proteasome. Based on their distinct biological functions, these 20S proteasome-associating proteins can be cataloged into different groups. 19S, 11S and PA200 are well known as 20S proteasome activators (Coux, Tanaka et al. 1996; Bochtler, Ditzel et al. 1999; Glickman and Ciechanover 2002). They bind to either bottom of 20S proteasome and activate it by establishing a channel for substrate to access the proteolytic core. Kinases and phosphatases belong to another group, which can regulate the 20S proteasome by post-translational modifications (Pereira and Wilk 1990; Castano, Mahillo et al. 1996; Feng, Longo et al. 2001). They have been discussed in previous section. Members of heat shock proteins (HSPs) have also been reported as 20S proteasome associating proteins (Conconi, Petropoulos et al. 1998; Conconi, Djavadi-Ohanian et al. 1999; Verma, Chen et al. 2000; Boelens, Croes et al. 2001). The significance of such associations are not well understood.

Consistent with the nomenclature, HSPs are proteins that are upregulated in the cell after a heat stress. Most of them are chaperones, which can prevent protein denaturation and/or help denatured protein refold. The main families of HSP include HSP100, HSP90, HSP70, HSP60 and

small HSPs (sHSPs). Proteins from different families vary both in sequence and functions. HSP60, HSP70 and HSP90 are important for protein folding and maturation. HSP60 exists as multimer and HSP70 as monomer and are both key chaperones involved in the folding of diverse proteins. HSP90 monomer is important to maintain the native conformation limited set of proteins, most of them are signaling proteins. sHSPs and HSP00 are primarily involved in stress response. The former prevents protein denaturation and the latter rescues proteins for aggregation (Rutherford 2003).

sHSPs family has multiple members: alpha crystallin A (α A crystallin), alpha crystallin B (α B crystallin), HSP 27, etc. They all have a homologous crystallin domain. α B crystallin and HSP27 expressed in the heart, while α A crystallin does not. α B crystallin over-expression protects the heart against ischemia insult (Martin, Mestril et al. 1997; Latchman 2001; Rutherford 2003; Taylor and Benjamin 2005).

PKC ϵ over-expression mouse line is a well-established cardio-protected model. Upon ischemia insult, this model shows significant reduction of cardiac infarction (Pass, Zheng et al. 2001). As part of the effects to elucidate the mechanism for this protection, Dr. Ping's group identifies 93 proteins within the PKC ϵ subproteome combining the power of immuno-precipitation, gel electrophoresis and mass spectrometry (Ping, Zhang et al. 2001; Vondriska, Klein et al. 2001). In subsequent research, several members within this subproteome were studied. Kinases, such as Lck, Src, Bmx, Erk; transcriptional factors, such as NF κ B, AP1; mitochondrial proteins, such as ANT, VDAC play roles in the protection (Ping, Zhang et al. 1999; Ping, Zhang et al. 1999; Li, Ping et al. 2000; Ping, Song et al. 2002; Zhang, Ping et al. 2003; Zhang, Ping et al. 2004). However, regulation of proteasome, the protein degradation machinery, was not covered by the previous study. The PKC ϵ subproteome study did not identify 20S proteasome subunits within these protein

complexes. This could be the result of these subunits having similar molecular weight as the IgG light chain, used in immuno-precipitation or their low abundance within these complexes. Nevertheless, one of the well-known 20S proteasome binding proteins, α B crystallin, was shown to be part of this subproteome.

This current study represents the first characterization of 20S proteasome within PKC ϵ over-expression, cardio-protected model. α B crystallin, as a stress-induced protein associating with both 20S proteasome and PKC ϵ , was screened for its potential as a 20S proteasome regulator in the heart.

Materials and Methods

Generation and Characterization of PKC ϵ Transgenic Mouse Lines

Standard techniques were used for the production and generation of PKC ϵ over-expression mouse. Briefly, a cardiac specific α -myosin heavy chain promoter was used to drive the expression of PKC ϵ cDNA mutants in FVB/N mice. An HA tag was inserted into the 5' end of the construct, which allowed differentiation of transgene expression from that of endogenous PKC ϵ . Constitutively active PKC ϵ (AE-PKC ϵ) is created by an A159E mutation at the pseudosubstrate domain. Mouse line expresses low level of the PKC ϵ transgenic protein and is inherently protected from cardiac ischemic injury. The phenotype of this transgenic mouse line has been previously characterized.

Purified Murine Heart 20S Proteasome

10g of mice heart tissue was homogenized by a potter homogenizer in homogenize buffer (Tris 20mM pH 7.8, EDTA 0.1mM, DTT 1mM, with protease inhibitor cocktail from Roche and phosphatase cocktails from Sigma). Cytosolic fraction was collected as the supernatant after 2hr centrifugation at 25,000g. Cytosolic fraction was then fractionated by ammonium sulfate precipitation. The pellet, collected between 40% to 60% ammonium sulfate saturation, was re-suspended in 10ml dialysis buffer (Tris 20mM pH 7.4, MgCl₂ 5mM, DTT 0.5mM). Re-suspended fraction was dialyzed again 4L dialysis buffer overnight to remove ammonium sulfate from it. The dialysate was fractionated with preparative-scale strong-anion-ion-exchange column (Q FastFlow resin packed in XK 26/40 column from GE healthcare) by stepwise salt concentration gradient elution at flow rate 5ml/min (45% B, until UV280nm monitor reading goes to baseline; 75% B, collect 200ml; 100% B, until UV280nm monitor goes to baseline. Buffer A: Tris 20mM pH 7.4, MgCl₂ 5mM, DTT 0.5mM, Glycerol 10%; Buffer B: Tris 20mM pH 7.4, MgCl₂ 5mM,

DTT 0.5mM, Glycerol 10%, KCl 600mM). 75% B fraction enriched in 20S proteasome. This fraction was centrifuged at 5°C 42,000rpm (Ti45 fixed angle rotor from Beckman) for 19hr. The pellet was collected, re-suspended in buffer A and resolved with analytical-scale strong-ion-exchange column (Mono Q HR5/50 from GE healthcare) with a linear salt concentration gradient from 0% B to 100% B through 17.5 column volume. The purified 20S proteasome was recovered in fractions around 60%B.

2D-electrophoresis and LC-MS/MS

2D electrophoresis was conducted using Bio-Rad 11cm apparatus. Firstly, purified 20S proteasome was desalted by TCA/Acetone precipitation. The desalted dry pellet was resuspended and resolubilized with IPG rehydration buffer (7M urea, 50mM DTT, 4% CHAPS, 0.2% 3-10 BioRad ampholytes). 11cm NL (non-linear) (BioRad) IPG was rehydrated in this solution overnight, and then isoelectrofocusing was conducted with BioRad IEF cell (250V, linear gradient 20 min, 250V, step and hold 5hrs, 3000V, linear gradient 1hr, 3000V, step and hold 1hr, 8000V, linear gradient 1hr, 8000V, step and hold for a total of 49375 Vhrs.). After IEF, proteins in IPG strips were reduced by 2% DTT solution and alkylated by 2.5% IAA sequentially for 10 minutes each. The second dimension electrophoresis was run with Bio-Rad pre-cast Criterion gel (12.5%) at 200V for 45min. The gel was fixed and stained with SYPRO Ruby protein dye (Molecular Probes).

Gel plugs stained by SYPRO Ruby were cut out with robotic gel cutter from Bio-Rad. Proteins within these spot were digested with trypsin (Promega). The resulting peptides were resolved with RP-HPLC column coupled online to a mass spectrometer (QSTAR from Applied Biosystems), which would be able to generate mass spectra and search them against protein database, identifying proteins within the original gel plugs.

SDS-PAGE and LC-MS/MS

SDS PAGE was performed with Bio-Rad Mini-Protean II apparatus according to classic Laemmli protocol using 12.5% poly-acrylamide gels. Electrophoresis was conducted at 120V DC for 1hr. After that, the gels were fixed and visualized by Colloidal Coomassie blue G-250 staining.

Gel plugs stained by Coomassie blue G-250 were cut out manually. Proteins within these spot were digested with trypsin (Promega). The resulting peptides were resolved with RP-HPLC column coupled online to a mass spectrometer (QSTAR from Applied Biosystems), which would be able to generate mass spectra and search them against protein database, identifying proteins within the original gel plugs.

Western-Blotting

Proteins resolved with SDS-PAGE were transferred in solution to nitrocellulose membrane (Pall Life Sciences, 0.45 μ M pore diameter). The transfer efficiency was checked by staining the blot with Ponceau S (BioRad), which could be removed by Tris-Buffered Saline with 1% Tween-20 (TTBS) washing. The remaining procedure was as follows: Block the transblot with 5% milk for 1 hour; Probe with 1st antibody (1000X dilution in 5% milk, 1% Tween-20) for 1 hour; Wash with TTBS (Tris-buffered solution with 1% Tween-20) for 3 times of 5 minutes each; Probed with HRP-linked 2nd antibody (3000X dilution in 5% milk, 1% Tween-20) for 1hr; Wash with TTBS for 3 times of 5 minutes each; Finally the transblot was incubated with ECL reagent (GE healthcare) for 1 minute and chemiluminescent signal recorded with film (Kodak).

Immuno-precipitation

Hearts taken from 8 weeks old PKC ϵ over-expression mice and wild type mice were homogenized in buffer contains protease cocktail and phosphatase cocktail with potter

homogenizer. Cytosolic fractions were collected by centrifuge at 100,000g for 1 hour. 300µg proteins from each of these fractions were pre-cleared by incubate with 20µl protein A/G beads from Santa Cruz for 30 minutes at 4°C. Supernatants were recovered and incubated overnight with 20µl protein A/G beads and 3µl primary antibody (0.25µg/µl) at 4°C. Beads were washed three times for 5 minutes each with 1ml TTBS. Beads were then boiled with 200ul SDS PAGE loading buffer for 5 minutes. Supernatants were collected and ready to use for subsequent SDS PAGE and western blot study.

Fluorescent Peptide Substrates and 20S Proteasome Activities Assay

Z-Leu-Leu-Glu-AMC from Sigma was used to measure β 1 subunit activity. (10X assay buffer: 250mM HEPES, pH 7.5; 5mM EDTA; 0.5% NP-40; 0.01% SDS). Boc-Leu-Ser-Thr-Arg-AMC from Sigma was used to measure β 2 subunit activity. (10X ssay buffer: 250mM HEPES, pH 7.5; 5mM EDTA; 0.5% NP-40; 0.01% SDS). Suc-Leu-Leu-Val-Tyr-AMC from Bachem was used to measure β 5 subunit activity. (10X assay buffer: 250mM HEPES, pH 7.5; 5mM EDTA; 0.3% SDS).

Proteolytic activities of 20S proteasome were compared among heart samples from different animal models (wild type mice, PKC ϵ over-expression mice). 10µg of protein from cytosolic fraction of murine heart homogenate was used. The specific proteasome inhibitor, Epoxomicin (Boston Biochem), was used in a parallel study as negative control for 20S proteasome activity assay.

Statistical Analysis

All data are presented as Mean \pm S.E. Groups are compared using two samples *t* test for unpaired data. A *P* value of less than 0.05 was considered significant.

Results

α B-Crystallin Co-purifies with 20S Proteasome from ICR Murine Heart

Purified murine cardiac 20S proteasome was resolved by 2D electrophoresis (Figure 16, Panel A). The α B crystallin was identified within the 2D gel as a 20S proteasome associating protein by mass spectrometry (QStar from Applied Biosystems). The spectra generated were searched against IPI database using Mascot search engine (Matrix Science). 50% of α B crystallin sequence was obtained and its N-terminal was determined to be acetylated (Figure 16, Panel B).

PKC ϵ Over-expression Mice have Increased Expression of 20S Proteasome Subunits;

While the Expression of 19S Subunits is Largely Unaffected

Proteasome subunits expression levels were compared between PKC ϵ over-expression mice and wild type control mice. This comparison was conducted with quantitative western blotting. The expression levels of the 20S proteasome subunits α 3, α 7 and β 5 were significantly up-regulated in PKC ϵ over-expression mice (Figure 17, Panel A); on the other hand, the expression levels of the 19S proteasome subunits Rpt1 and Rpn2 showed no significant changes (Figure 17, Panel B).

20S Proteasome Peptidase Activities are not Significantly Altered in

PKC ϵ Over-expression Mice

Peptidase activities of 20S proteasome within cytosolic fractions were compared among PKC ϵ over-expression mice and wild type control mice. The 20S proteasome specific inhibitor was used to minimize proteolytic activities contributed from other intracellular proteases. Despite the higher population of 20S proteasome subunits within PKC ϵ over-expression mice, all three peptidase activities of 20S proteasome showed no significant difference over wild type control

mice (Figure 18).

α B-Crystallin Expression is Upregulated in PKC ϵ Over-expression Mice

α B crystallin expression level was compared between PKC ϵ over-expression mice and wild type control mice with western blot. In the cytosolic fraction, PKC ϵ over expression mice showed small but significant up-regulation of α B crystallin (Figure 19).

α B-Crystallin Immunoprecipitation, 20S Proteasome Western Blotting

The association between α B crystallin and 20S proteasome was compared between PKC ϵ over-expression mice and wild type control mice by immuno-precipitate α B crystallin containing protein complexes followed with western blot α 7 subunit of 20S proteasome. The chemiluminescent signal was significant stronger in PKC ϵ over-expression mice compare to the wild type mice (Figure 20), which suggests more 20S proteasome and α B crystallin association in the transgenic mice.

20S Proteasome Immunoprecipitation, PKC ϵ Western Blotting

The association between PKC ϵ and 20S proteasome was also compared between PKC ϵ over-expression mice and wild type control mice. This was conducted by immuno-precipitation of the 20S proteasome containing protein complexes followed with western blotting with PKC ϵ . In the PKC ϵ over expression mice, more PKC ϵ was present in the 20S proteasome containing protein complexes (Figure 21).

Discussion

α B-Crystallin Associates with 20S Proteasome

Consistence with previous study in other tissues, α B crystallin was also found to co-purify with 20S proteasome from murine heart. In an *in vitro* study, $\alpha 7$ was suggested to be the subunit of 20S proteasome interacting with α B crystallin. This study in the heart provided further evidence that the interaction between 20S proteasome and α B crystallin are ubiquitous among tissues.

As reported by Dr. Conconi (Conconi, Petropoulos et al. 1998; Conconi, javadi-Ohaniance et al. 1999), purified latent rat liver 20S proteasome can be inhibited by α B crystallin, while activated liver 20S proteasome can not. We were unable to reproduce this using *in vitro* biochemical experiment with murine cardiac 20S proteasome. This could be due to the difference in the biochemical properties between rat liver 20S proteasome and murine cardiac 20S proteasome, or most likely due to variations in purification protocols. Nevertheless, the association between 20S proteasome and α B crystallin has been confirmed by immuno-precipitation experiments of this study.

In PKC ϵ Overexpression Mice, the Expression Level of 20S Proteasome Subunits

are Upregulated, but the Total 20S Proteasome Peptidase Activities

do not Change Significantly

This study presented the first characterization of proteasome regulation in a cardio-protected phenotype, PKC ϵ over-expression mouse line. Ubiquitin-proteasome system is responsible for the turnover of the majority of intracellular proteins, among them, including lots of transcriptional factors, signaling proteins. Proteasome is the final executer of this pathway. The contribution of its regulation to accommodate a cardio-protected phenotype is sure of great significance.

Three 20S proteasome subunit expression levels have been shown to be up-regulated in this cardio-protected model; while the expression level of two 19S proteasome subunits showed no significant difference. This is consistent with the fact that 20S proteasome subunits and 19S proteasome subunits are under different transcriptional control mechanisms. Activation of NF κ B has been known leading to the up-regulation of 20S proteasome. Dr. Ping's group has reported NF κ B activation as one of the result of PKC ϵ expression (Li, Ping et al. 2000). Even the 20S proteasome subunit expression levels were increased in transgenic mice, its peptidase activities showed no significant difference in non-stressed condition compare to the wild type mice. This suggested there is an inhibitory mechanism within the transgenic model in such condition.

**α B-Crystallin is a Key Stress-response Protein Regulated in PKC ϵ Overexpression Mice,
Which Associates with 20S Proteasome**

α B crystallin is a member of sHSP family expression in the murine heart. Ubiquitin, key component of ubiquitin-proteasome system, is also a heat-shock protein. α B crystallin is a chaperone and its over-expression has been shown to be protective upon cardiac ischemic insult. Indeed, in the cardio-protected transgenic mice, under non-stressed condition, there is a small but significant up-regulation of α B crystallin expression level. Its protective effect has been attributed to its ability to prevent protein denaturation. Even α B crystallin has been well known as 20S proteasome associating protein, its potential of regulating 20S proteasome under stress has not been tested.

As the first step to study the effect of α B crystallin regulating cardiac 20S proteasome in resisting ischemic insult, their association has been compared between cardio-protected model and wild type control. There are more 20S proteasome associating with α B crystallin in the

protected model. Previous reports documented latent 20S proteasome could be inhibited with α B crystallin, which would partially explain 20S proteasome inhibition in the protected model. Such effect was not reproduced *in vitro* with purified cardiac 20S proteasome. This could be due to the difference between the two 20S proteasome purification protocols.

In the Cardio-protected Mice, More PKC ϵ Associates with 20S Proteasome

Immuno-precipitation study showed more PKC ϵ was recruited to 20S proteasome containing protein complexes in the cardio-protected model. In the purified murine cardiac 20S proteasome preparation, however, PKC ϵ was not shown as 20S proteasome associating proteins. α B crystallin, as a well-known 20S proteasome associating protein, is recruited to PKC ϵ subproteome in the cardio-protected model. As reported in previous section, there are meanwhile significantly more 20S proteasome associating with α B crystallin in the cardio-protected model. This supported the hypothesis proteins within PKC ϵ subproteome regulated 20S proteasome activity. The availability of α B crystallin mice enables us to test this in the near future.

Summary

20S proteasome associating proteins have impacts on its functions. α B crystallin is member of sHSP family, well known associating with 20S proteasome. This has been confirmed by this study in the murine heart. Significantly, α B crystallin has also been reported playing a significant role in cardio-protection against ischemia/reperfusion injury.

PKC ϵ over-expression mouse is a well-established cardio-protected model. Since it was generated, extensive studies have been conducted to uncover the mechanisms that are responsible for this phenotype. Several key proteins have been identified. These included signaling proteins, transcriptional factors and apoptosis related proteins. However, protein degradation and stability control pathway, specifically proteasome and heat shock proteins, have been largely overlooked. This study represented a first report in this area.

In the transgenic cardio-protected mouse, the expression levels of 20S proteasome subunits were significantly induced, while those of 19S proteasome subunits were un-affected. NF κ B has been showed as the transcriptional factor that induces the expression of 20S proteasome subunits. This is consistence with previous report from Dr. Ping's group that NF κ B was activated in this transgenic model (Li, Ping et al. 2000). In spite of enhanced 20S proteasome expression level, its peptidase activities showed no significant increase in transgenic mice. There must be certain mechanism prevented this augment in transgenic mice.

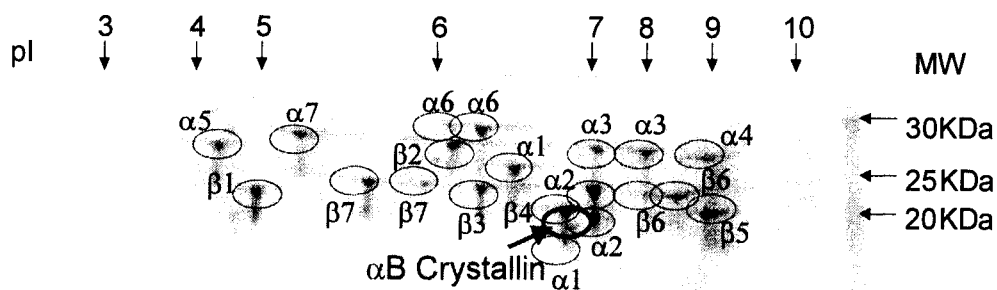
α B crystallin has been reported as a 20S proteasome associating protein that could inhibit its activity. The study showed only latent 20S proteasome, which is its *in vivo* conformation, could be inhibited by α B crystallin, while the activated counterpart was not affected by α B crystallin. This inhibitory effect was not reproduced with murine cardiac 20S proteasome. This could be explained by that purified 20S proteasome was in an activated state. Nevertheless, the association between

20S proteasome and α B crystallin was significantly enhanced in the transgenic model. In the transgenic model, α B crystallin was recruited to PKC ϵ subproteome compare to the wild type. In this study, we have also seen increased association between 20S proteasome and PKC ϵ . Proteins within PKC ϵ subproteome could be responsible for the inhibition of 20S proteasome in the heart. The availability of α B crystallin transgenic mice would enable us to clarify this phenomenon in the near future.

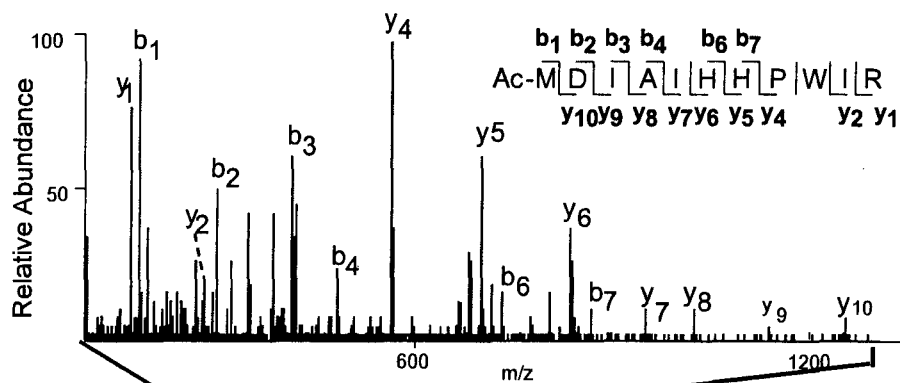
Figure 16. α B-Crystallin Co-purifies with 20S Proteasome from ICR Murine Heart. Panel A.

Purified murine cardiac 20S proteasome was resolved by 2D electrophoresis using Bio-Rad apparatus. 2D gel was fixed, stained with SYPRO Ruby protein dye and image recorded with Bio-Rad fluorescent imager. All spots shown on the gel were cut and proteins within them identified with Qstar LC/MS/MS from Applied Biosystems. **Panel B.** Spectrum of α B-Crystallin N-terminal peptide acquired with Qstar LC/MS/MS, which is shown as underlinedD bolded red character within its sequence. All other peptides with spectra recorded with the mass spectrometer were shown as red underlined characters.

Panel A.



Panel B.

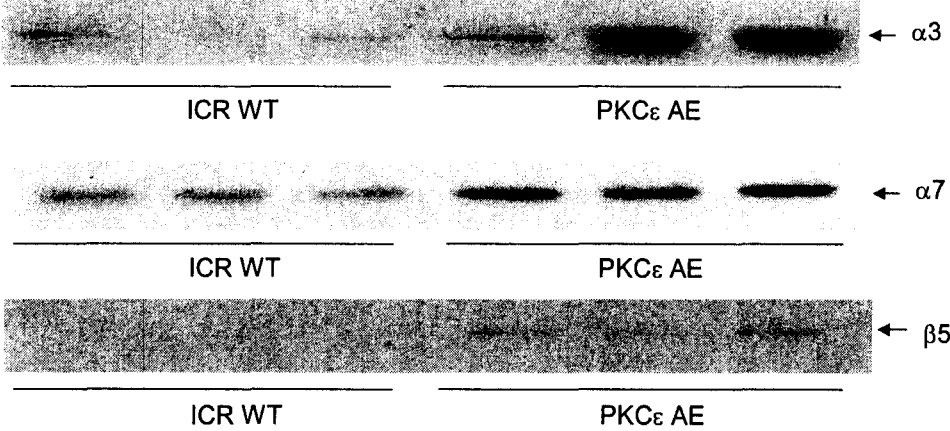


1 MDIAIHPWI RRPFFPFHSP SRLFDQFFGE HLLESDFST ATSLSPFYLR
 51 PPSFLRAPS~~W~~ IDTGLSEMRL EKDRFSVNL~~D~~ VKHFSPEELK VKVLGDVIEV
 101 HGKHEERQDE HGFISREFHR KYRIPADVDP LTITSSLS~~SD~~ GVLTVNGPRK
 151 QVSGPERTIP ITREEKPAVA AAPKK

Figure 17. PKC ϵ Over-expression Affects Proteasome Subunits Expression Levels.

Proteasome subunit expression levels in the cardiac tissue cytosolic fractions were compared between PKC ϵ over-expression mice and wild type control mice by western blot. **Panel A.** 20S proteasome subunits, α 3, α 7 and β 5, were all upregulated in the transgenic mice. **Panel B.** Expression levels of 19S proteasome subunits, Rpt1 and Rpn2, showed no significant difference between transgenic mice and non-transgenic mice in non-stressed condition.

Panel A.



Panel B.

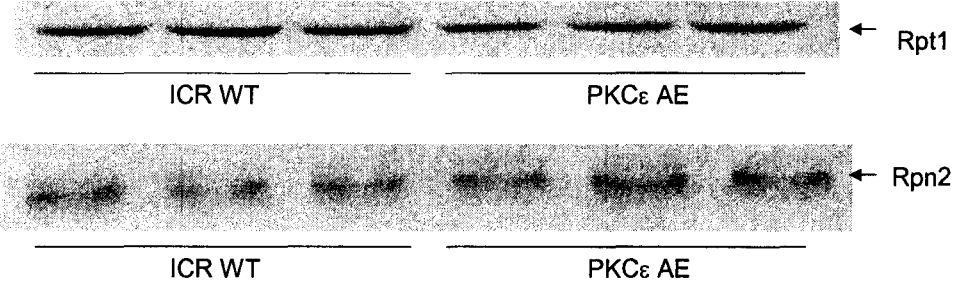


Figure 18. 20S Proteasome Peptidase Activities are not Significantly Altered in PKC ϵ

Over-expression Mice. 10 μ g of cytosolic fraction of heart homogenate were used in the proteasome activity assays. LLE-AMC was used as substrate to evaluate β 1 activity; LSTR-AMC was used to evaluate β 2 activity; LLVY-AMC was used to evaluate β 5 activity. After adding substrates, the reaction mixtures were incubated 1hr at 37°C. The free AMC fluorescence was measured using a fluorometer (excitation 390nm; emission 450nm). Parallel experiments were conducted use 20S proteasome specific inhibitor, epoxomicin, to minimize background. * $p < 0.05$ compare to WT. n=3 in all experiments. Data are expressed as mean \pm S.E.

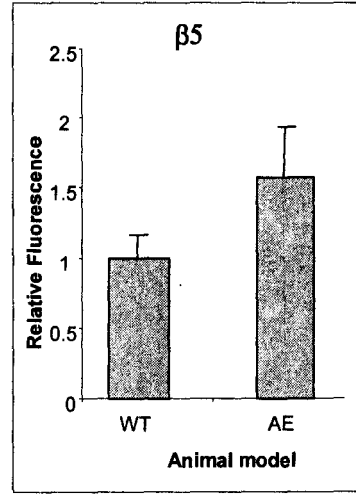
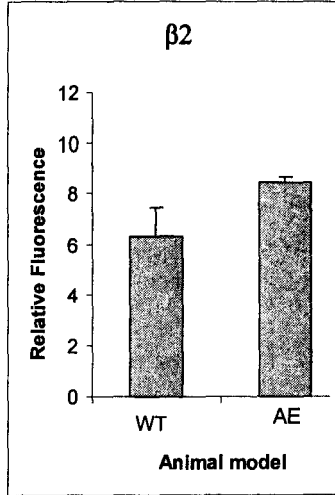
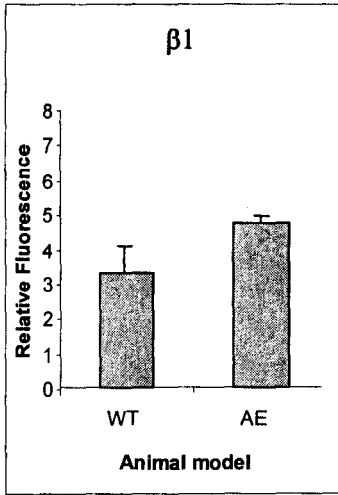


Figure 19. α B-Crystallin Expression is Up-regulated in PKC ϵ Over-expression Mice.

Cytosolic fractions were recovered from supernatant after 1hour centrifugation at 100,000g. The expression level of α B-Crystallin was compared among PKC ϵ over-expression mice and control mice. Transgenic mice showed small but statistically significant up-regulation of α B-Crystallin expression level.

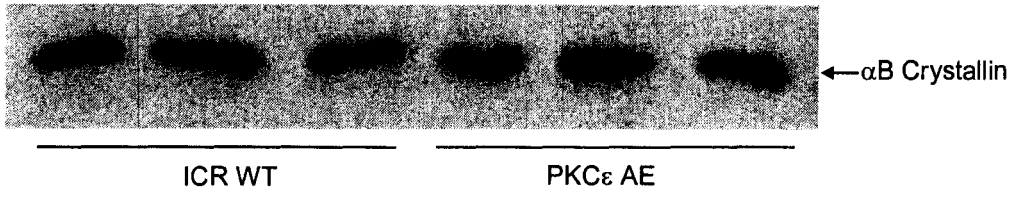


Figure 20. Comparison of α B-Crystallin, 20S Proteasome Association. Association of α B crystallin and 20S proteasome between PKC ϵ overexpression mice and wild type control mice was compared by immuno-precipitating α B crystallin containing protein complexes from the cytosolic fraction followed by western blotting against the 20S proteasome α 7 subunit . Transgenic mice showed significantly higher association.

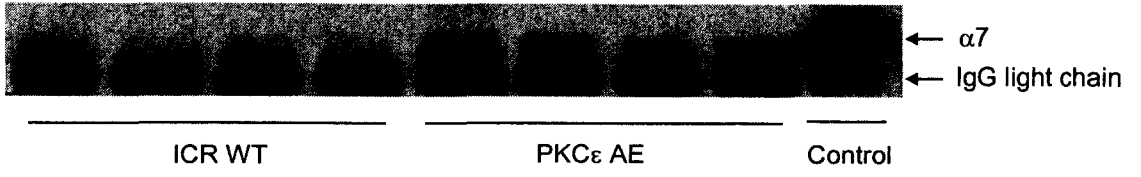
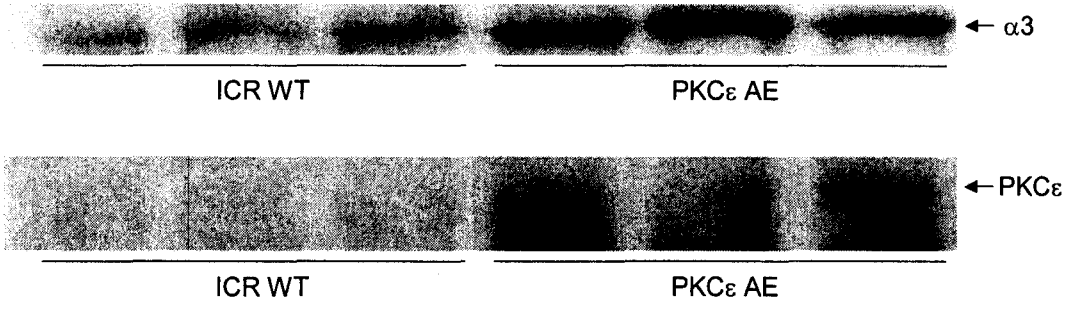


Figure 21. Comparison of PKC ϵ , 20S Proteasome Association. Association of α B crystallin and 20S proteasome between the PKC ϵ over-expression mice and wild type control mice was compared by immuno-precipitating 20S proteasome containing protein complexes from the cytosolic fraction followed with PKC ϵ western blotting. Transgenic mice showed significantly higher association.



CHAPTER V

SUMMARY AND DISCUSSION

The completion of Human Genome Project, the biggest achievement in basic biological science within the last decade, provided us a blue print of human being, however, we are still far from fully understanding it. Proteins serve as building blocks of life. They functions as cell skeletons, enzymes, transporters, ion channels and hormones. Compared to ~30,000 genes identified and sequenced from Human Gnome Project, there are far more proteins being generated by human, as results of alternative splicing and post-translational modifications. Proteins are regulated at multiple levels, including alternative splicing, transcriptional control, translational control, post-translational control, translocation and degradation. This thesis represents a first comprehensive characterization of major component within murine cardiac protein degradation pathway, 20S proteasome: composition, function and regulation.

Intracellular protein turnover mainly operates through two systems. The well-known lysosome pathway, turnovers membrane proteins and pathogens. They are enclosed in a vesicle structure called endosome. The fusion of endosome with lysosome exposes its content to lysosomal proteases. The majority of nuclear and cytosolic proteins are turned over by proteasome related pathways into peptides. These peptides are either transferred to cell surface as antigens or further degraded by Tripeptidyl Peptidase II (TPP II) into amino acid to replenish cytosolic pool. Several

proteasome-involved protein degradation mechanisms have been proposed. The elucidation of poly-ubiquitin tagging pathway won Nobel Prize in Chemistry 2004. Ornithine decarboxylase and cyclin D1 are targeted for 26S proteasome after binding to antizyme. Protein degradation by 26S proteasome independent of both ubiquitin and antizyme has also been reported. 20S proteasome is known to degrade oxidative damaged proteins. It can turn over native proteins at *in vitro* setting.

Regulation of proteasome protein degradation process is complex. The regulation can be accomplished selectively at protein substrate level. Its ubiquitination, post-translational modification and binding partner, impact its half-life. This regulation can also be achieved at proteasome level. Its post-translational modification, associating partner and assembly determine its proteolytic activities and selectivity towards groups of substrates.

Establishment of large-scale 20S proteasome purification protocol from murine heart is a pre-requirement for its characterization. Considering the nature of sample, cost, possibility of genetic manipulation, biochemical method is optimal. The high stability, high molecular weight and highly charged properties of 20S proteasome were utilized along the purification procedure to reach a final purity higher than 95%. Moreover, 20S proteasome in the preparation has been proved to be structurally intact and functionally active. Purified 20S proteasome was analyzed by mass spectrometer after in-solution digestion or in-gel digestion following gel electrophoresis, including 1D SDS PAGE, 2D electrophoresis and blue native gel electrophoresis. All these approaches have been proven to be complementary. 1D SDS PAGE provided higher reproducibility, higher sample loading capacity and possibility of analyzing all proteins resolved throughout one lane. 2D electrophoresis separated all 20S proteasome subunits, including

post-translational modified subunits across the gel. This enabled us to analyze each subunit individually and provides unique advantage when to identify post-translational modified residues. Blue native gel electrophoresis resolved populations of 20S proteasome containing protein complex while keeping them intact. Different species of 20S proteasome and its components could be studied independently. Direct in-solution digestion skipped peptides extraction step, which was mandatory for in-gel digestion procedure. High hydrophobicity peptides were difficult to extract. In-solution digestion overcame this bias. As a result, all 17 subunits of 20S proteasome have been identified, including 14 constitutive and 3 inducible subunits. Catching unique sequence within a protein was sufficient for protein identification, however a high sequence coverage by mass spectrometer was necessary for post-translational analysis. The pI of murine cardiac 20S proteasome was 0.8 units lower than theoretical value, which suggested the existence of post-translational modifications. Several subunits were represented with multiple focused spots in 2D gel. This fortified the idea that 20S proteasome was post-translational modified.

20S proteasome were phosphorylated endogenously. Both immuno-blotting and mass spectrometric analysis showed purified 20S proteasome subunits were phosphorylated. As a first study of such modifications, phosphorylations were removed globally by CIAP. CIAP treatment enhanced $\beta 2$ and $\beta 5$ subunits activities significantly. Within *in vitro* system, CIAP could also be degraded by 20S proteasome serving as competitive peptidase assay inhibitor. PP2A, PKA and CKII have been identified as murine cardiac 20S proteasome associating enzymes. PKA and CKII could phosphorylate 20S proteasome to enhance its peptidase activities, while serine/threonine phosphatase, PP2A, was a negative regulator of 20S proteasome activities.

20S proteasome associating proteins can also regulate 20S proteasome. In PKC ϵ over-expression transgenic mice, 20S proteasome subunits, represented by α 3, α 7 and β 5, were up-regulated. However, 19S subunits, represented by Rpt1 and Rpn2, showed no change. Surprisingly, up-regulation of 20S proteasome subunits expression levels did not lead to its significant activity change. 20S proteasome subunit and 19S subunits are under different regulatory control. Dr. Ping's group reported PKC ϵ activation induces NF κ B, which has been reported as 20S proteasome transcriptional activator. These over-expressed 20S proteasome existed in a suppressed state to maintain an unchanged overall 20S proteasome activity in the cytosol. α B crystallin is a small heat shock protein associating with and inhibiting 20S proteasome. This association has been confirmed with heart sample in this study. α B crystallin is also a component of PKC ϵ subproteome. In the PKC ϵ over-expression transgenic model, it is recruited to PKC ϵ subproteome compared to wild type control. In the transgenic model, increased association of 20S proteasome with α B crystallin and PKC ϵ subproteome was evidenced with co-immuno-precipitation study.

α B crystallin knockout mice will be used as an important model to further study the functional impact of this small heat shock protein over 20S proteasome. Murine cardiac 20S proteasome from the transgenic models will be obtained and compared with that from the control model at proteomic level. The regulation of relative population of proteasome species and its effect toward phenotype are other important directions to follow.

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Peer-reviewd Articles:

Zong C, Qiao J, Gomes A, Berhane B, Glen Y, Edmondson R, Jones R, Ping P. Phosphorylation is a Major Regulator of the Cardiac 20S Proteasome. (In preparation)

Gomes A, **Zong C**, Edmondson R, Berhane B, Wang G, Le S, Young G, Zhang J, Vondriska T, Whitelegge J, Jones R, Joshua I, Thyparambil S, Pantaleon D, Qiao J, Loo J, Ping P. The Murine Cardiac 26S Proteasome—An Organelle Awaiting Exploration. *Annals of the New York Academy of Sciences*. 2005;1047:197-207.

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4. Aldrin V. Gomes, **Chenggong Zong**, David A. Liem, Beniam Berhane, Peipei Ping. Role of the Ubiquitin-Proteasome System in Ischemia/Reperfusion Injury. *Keystone Symposia Ubiquitin and Signaling, New Mexico*. 2005

5. **Nobel (Chenggong) Zong**, Beniam Berhane, Joe X. Qiao, Tomas M. Vondriska, Joseph A. Loo, Peipei Ping. Isolation, Purification, and Functional Proteomic Characterization of the Murine Myocardial 20S Proteasome. *Molecular Cellular Proteomics*. 2004, Vol 3;S7.

6. Zhang J, **Zong C**, Vondriska TM, Qiao XJ, Cardwell EM, Ping P. Proteomic Mapping of Cardiac Protective Signaling Complexes Using Blue Native Electrophoresis and Bioinformatic Modeling. *FASEB J*. 2003, Vol.17:A103.