Mammalian Proteasome Subpopulations with Distinct Molecular Compositions and Proteolytic Activities

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Running title: Mammalian Proteasome Subpopulations

Abbreviations: BisTris Bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methan, EPPS N-(2-hydroxyethyl)piperazine-N'-(3-propanesulfonic acid), FFE free flow electrophoresis, HPMC (Hydroxypropyl)methyl cellulose, PTM posttranslational modification, SPADNS Sulfanilic acid azochromotrop, UPS ubiquitin-proteasome system
Summary:

The proteasome-dependent protein degradation participates in multiple essential cellular processes. Modulation of proteasomal activities may alter cardiac function and disease phenotypes. However, cardiovascular studies reported thus far, yielded conflicting results. We hypothesized that a contributing factor to the contradicting literature may be caused by existing proteasome heterogeneity in the myocardium. In this investigation, we provide the very first direct demonstration of distinct proteasome subpopulations in murine hearts. The cardiac proteasome subpopulations differ in their molecular compositions and proteolytic activities. Furthermore, they were distinguished from proteasome subpopulations identified in murine livers. The study was facilitated by the development of novel protocols for in-solution isoelectric focusing of multi-protein complexes in a laminar flow, which support an average resolution of 0.04 pH units. Utilizing these protocols, the majority of cardiac proteasome complexes displayed an isoelectric point of 5.26, with additional subpopulations focusing in the range from pH 5.10-5.33. In contrast, the majority of hepatic 20S had a pl of 5.05 and focused from pH 5.01-5.29. Importantly, proteasome subpopulations degraded specific model peptides with different turnover rates. Among cardiac subpopulations, proteasomes with an approximate pl of 5.21 showed 40% higher trypsin-like activity than those with pl 5.28. Distinct proteasome assembly may be a contributing factor to variations in proteolytic activities, since proteasomes with pl 5.21 contained 58% less of the inducible subunit β2i compared to those with pl 5.28. In addition, dephosphorylation of 20S demonstrated that besides molecular composition, posttranslational modifications largely contribute to its pl. These data suggest the possibility of mixed 20S proteasome assembly, a departure from the currently hypothesized two subpopulations: constitutive and immuno-forms. The identification of multiple distinct proteasome subpopulations in heart provides key mechanistic insights for achieving selective and targeted regulation of this essential protein degradation machinery. Thus, proteasome subpopulations may serve as novel therapeutic targets in the myocardium.
Introduction:

The half-life of many proteins in cell proliferation, apoptosis and gene expression is dependent on the ubiquitin-proteasome system (UPS) [1]. In fact, up to 90% of intracellular proteins in growing mammalian cells are degraded by the UPS [2]. Proteasome inhibitors have been proven to be remedies in attenuating oncogenesis [1]. Recently, the applications of proteasome inhibitors were also explored in cardiovascular systems, however, the results have been conflicting [3-5]. Subsequent analysis of the UPS in cardiac tissue revealed co-expression of constitutive and immunoproteasome subunits [6, 7], which raises the question whether proteasome diversity contributes to the current controversies.

In rat skeletal muscle, the presence of proteasome heterogeneity was suggested [8, 9]. Conventionally, two proteasome complexes are currently accepted in UPS models [10, 11]. In these models, the 26S proteasomes recognize, unfold, and finally degrade polyubiquitinated proteins into short peptides [12-14]. The degradation is catalyzed by the 20S core complexes, which associate with one or two of the activating complexes 19S, 11S and PA200 [15, 16]. The proteolytic activities of the 20S are described as caspase-, trypsin- and chymotrypsin-like [17, 18]. The proteolytic active sites are located on the proteasome subunits $\beta_1$, $\beta_2$ and $\beta_5$ [19-21]. In total, 14 $\alpha$ and 14 $\beta$ subunits constitute 20S proteasomes, forming 4 stacked rings with seven unique $\alpha$ subunits in the two outer and seven unique $\beta$ subunits in the two inner rings [22, 23]. A second subpopulation with distinct proteolytic activities is assembled when $\beta_1$, $\beta_2$ and $\beta_5$ are substituted by $\beta_{1i}$, $\beta_{2i}$ and $\beta_{5i}$ upon induction by interferon-gamma in mammals [24, 25]. Remarkably, co-expression of inducible $\beta$ subunits has been reported for certain cells and tissues without preceding induction or inflammation [26-29]. These findings introduced the hypothesis that mammalian cells potentially contain two different types of 20S proteasome complexes: the constitutive and the immuno-form. Additional 20S subtypes were suggested by Dahlmann et al. [8, 9], but further evidence is imperative to establish their existence in universally effective UPS models.
Proteomic technologies have been essential in characterizing the UPS in the past [6, 7, 26-30]. However, it is hitherto unknown in what manner 20S subunits can be assembled besides the constitutive and the immuno-form. Analytical high resolution chromatography of native 20S from rat skeletal muscle suggested the existence of intermediate 20S complexes, composed of both constitutive and inducible β subunits [8, 9]. The molecular compositions of 20S proteasomes have been recognized to play a pivotal role in its proteolytic activities [24, 25, 31]. Individually altered expression of subunits bearing the proteolytic sites significantly impacted the 20S proteasome peptidase activities [32, 33]. Therefore, proteasome diversity translates into alternate function. These studies suggest that the current view on proteasome systems (the constitutive and inducible models) may no longer adequately describe the 20S proteasome complexity.

In the present study, the hypothesis whether proteasome subpopulations exist in other mammals was investigated in *Mus musculus*. With regard to current controversies in cardiovascular proteasome research, we addressed the question whether distinct proteasome complexes exist in cardiac tissue. Previous studies from our and other groups indicated charge differences between subpopulations by different assembly and posttranslational modifications (PTMs). Therefore, separation according to the isoelectric point (pI) appeared to be essential. Native isoelectric focusing (IEF) at high resolution of multiprotein complexes in excess of 700kDa, such as the 20S, is quite challenging. Free flow electrophoresis (FFE) was chosen for this task, because of its proven ability to separate native proteins as well as organelles in a system without passing the analytes through solid matrix [34-36]. In our study, a novel FFE protocol was employed for IEF to effectively separate 20S proteasome complexes.
Experimental Procedures:

Purification of 20S complexes
Proteasome complexes were purified from murine liver and hearts according to modified published protocols [6, 37]. Briefly, hearts or livers were homogenized in 20 mM Tris (pH 7.8), 0.1 mM EDTA, 1 mM DTT, complete protease inhibitors (Roche, Indianapolis, IN, USA), phosphatase inhibitor cocktails 1 and 2 (Sigma, St. Louis, MO, USA), and centrifuged at 25,000xg for 2h. Next, ammonium sulfate was added slowly to the supernatant at a final concentration of 40% and then centrifuged at 12,000xg for 1h. The procedure was repeated with the supernatant to achieve a final concentration of 60% ammonium sulfate, and again centrifuged at 12,000xg for 1h. The pellet was dissolved in 20 mM Tris (pH 7.4), 5 mM MgCl₂ and 1 mM DTT and dialyzed against the same solution over night. Then, the dialyzed sample was reconstituted to 45% B (A: 20 mM Tris, pH 7.4, 5 mM MgCl₂, 0.5 mM DTT, 10% glycerol; B: same as A plus 600 mM KCl) and loaded on a XK 26 40 column (GE Healthcare, Uppsala, Sweden) packed with Q Sepharose (GE Healthcare), which was equilibrated with 45% B. After washing the column, B was increased to 75%, the eluant collected, and centrifuged at 205,000xg for 19h. The pellet was dissolved by homogenization in buffer A and centrifuged at 3,000xg to remove insoluble particles. Then, the supernatant was loaded on a Mono Q 5/50 (GE Healthcare), equilibrated with A. Finally, purified 20S proteasome was eluted at approximately 55% B by running a shallow gradient up to 100% B. All steps were performed at 4°C or on ice.

Dephosphorylation of 20S
Partial dephosphorylation of 20S proteasome complexes was achieved by calf intestinal alkaline phosphatase treatment (CIAP, Promega, Madison, WI, USA). In variation with the 10x buffer supplied by the provider, dephosphorylation was performed in buffer lacking spermidine, thus containing 0.5 M Tris (pH 9.3), 10 mM MgCl₂ and 1 mM ZnCl₂. In total, 60 μg purified 20S proteasome was incubated in presence of 250 CIAP units for 30 min at 37°C.
Isoelectric focusing using free flow electrophoresis

Free flow electrophoresis was performed on the BD FFE System (BD, Franklin Lakes, USA). For high resolution FFE, a 0.4 mm spacer has been used, resulting in a total volume of 20ml in the separation chamber. Accurate and reproducible determination of the isoelectric point of the 20S proteasome complexes in narrow pH gradients was achieved by interval FFE. The separation chamber was tempered at 10°C to prevent protein degradation and precipitation. Furthermore, the device was operated in a cold room at 4°C to keep the samples in their latent state for high reproducibility in subsequent analysis of fractions by proteolytic assays. The BD FFE System features in total 7 inlets for the separation media. The outermost inlets were used for stabilization media as recommended by the manufacturer.

Stabilizing media were prepared dependent on the pH gradient used for IEF. For pH 3-10, 100 mM H₂SO₄ and 100 mM NaOH were used as anodal and cathodal stabilizer, respectively. The remaining five inlets were used for the separation media, containing 1% Servalytes 3-10. For pH 4-6, the anodal stabilizer solution contained 100 mM H₂SO₄, 50 mM acetic acid, 200mM 2-aminobutyric acid and 50mM glycylglycine. The cathodal stabilizer consisted of 100 mM NaOH, 50 mM Tris, 30 mM BisTris, 150 mM EPPS, 50 mM HEPES and 30 mM MOPS. The separation medium on the inlets 2-6 contained 1.2% Servalytes 4-6 and 0.3% Servalytes 3-10. All media used for separation, stabilization as well as counter flow contained 0.2% HPMC and 25% glycerol. In addition, 1 mM DTT was added to all media. Anodic and cathodic electrode solutions were prepared according to the manufacturer, containing 100 mM H₂SO₄ and 100 mM NaOH, respectively.

Samples subjected to FFE were diluted 1:10 with separation media. In general, 200 µl of diluted sample was applied, containing typically 60 μg purified 20S proteasome complexes. For visualization of injection, separation and fractionation, 1.5 µl SPADNS (1% w/v, Sigma) was added to each sample. The diluted samples were injected to the separation chamber at the cathodic sample inlet, using flow rates of 1ml/h for continuous and 9ml/h for interval mode with the improved separation protocol. Before and after sample entry, undiluted separation media was injected to the separation chamber at the same flow rate as the sample flow rate.
In continuous mode, the flow rate of the separation medium was set to 60ml/h and IEF conditions limited to 1500V and 50mA. For high resolution separation in narrow pH gradients, the media flow rate for sample entry was 300 ml/h. Then, prolonged IEF was performed at maximal 1200V and 30mA in interval mode with intervals set to 5 minutes and a reduced flow rate of 50 ml/h. Operating the FFE in interval mode means to virtually elongate the separation chamber and thus extending the residence time of the sample by alternating the pump direction of the media flow after each interval period between forward and backward. Accurate and reproducible IEF of 20S complexes was achieved after one hour in interval mode. Finally, the media flow rate was set to 300 ml/min to move the sample to the fraction collector. In both, the continuous as well as the interval mode, fractions were collected in 96 well plates when the red marker SPADNS reached the fraction collector.

**Separation of proteins by one or two-dimensional electrophoresis**

Isoelectric focusing of the FFE fractions was performed on the IPGphor (GE Healthcare) in combination with the Manifold (GE Healthcare). The samples were separated on pH 3-10, 18cm, IPG strips (GE Healthcare) according to a modified protocol of Gorg et al. [38]. The IPG strips were rehydrated in 350 μl buffer containing 8M (w/v) urea, 1% (w/v) CHAPS, 0.2% (w/v) DTT and 0.5% (v/v) Pharmalytes 3-10. Previous to IEF, FFE fractions were diluted at a ratio of 1:5 with buffer containing 9M (w/v) urea, 4% (w/v) CHAPS, 2% (w/v) DTT and 1% (v/v) Pharmalytes 3-10. Sample entry was facilitated by 30min at 150V, 30min at 300V and 30min at 600V. After that, the voltage was increased to 8000V using a 30min voltage gradient and IEF reached a steady state after a minimum of 18 kVh. Second dimension was performed on 12% AA gels after equilibration of the samples as published in [39]. Finally, the separated proteins were visualized by silver staining.

For one dimensional SDS-PAGE, FFE fraction were diluted with SDS sample buffer and separated on 12.5% SDS gels. Gels were either stained with silver or SYPRO Ruby (Invitrogen) according to the manufacturer. Fluorescence was detected by laser densitometry using a Typhoon 9600 variable mode imager (GE Healthcare) and quantified by the ImageQuant 5.2 software (GE Healthcare).
Blue native gel electrophoresis of FFE fractions was carried out according to a modified protocol of Schagger et al. [40]. FFE fractions were mixed with BN-sample buffer and separated on 6% acrylamide gels. Ferritin complexes (MW 440kDa) and thyrolobulin (MW 669 kDa; GE Healthcare) were used as molecular weight standards.

**Immunodetection of 20S subunits**

Immunoblotting was performed according to standard protocols. Proteasome subunit α3 was detected using customized polyclonal antibodies raised in rabbit at a dilution of 1:10,000. All other antibodies were obtained from Biomol, Plymouth, PA, USA, and used at dilutions from 1:1500 to 1:2000. HRP-conjugated goat-anti-mouse (Sigma) and goat-anti rabbit (BD) were used as secondary antibodies at a dilution of 1:5000. ECL detection (GE Healthcare) was performed prior to laser densitometry and quantification using the Scion Image software (NIH).

**20S proteasome activity assays**

Proteasome assays after IEF-FFE of purified 20S were measured using Z-LLE-AMC for caspase-like (Calbiochem, La Jolla, CA, USA), Boc-LSTR-AMC for trypsin-like (Sigma) and Suc-LLVY-AMC for chymotrypsin-like activities (Boston Biochem, Cambridge, MA, USA) [41]. All assays were performed in tri- or quadruplicates, using 5µl or 0.1µg of fractionated 20S complexes, typically 100 µM substrate, 25 mM HEPES (pH 7.5), 0.5 mM EDTA and 0.03% SDS for the caspase- and trypsin-like activities, or 0.05% NP-40 and 0.001% SDS for the chymotrypsin-like activity [6]. The substrate decomposition was monitored up to 90 min by excitation at 380nm and scanning the emission at 460nm every 15 min using the FluoroskanAscent fluorometer (Thermo, Waltham, MA, USA).
Results:

Native IEF of 20S proteasome complexes purified from mouse liver
Since the size of the native 20S proteasomes exceeds 700kDa, FFE was chosen for native IEF of multi-protein complexes. The choice originated from the assumption that the absence of a solid matrix, such as polyacrylamide, would enable high resolution IEF of protein complexes with high molecular weight. In initial experiments, conventional FFE in continuous mode was utilized to focus hepatic 20S proteasome complexes in the pH range from 3-10 (Fig. 1). Subsequently, the fractions were mixed with SDS buffer and separated by SDS-PAGE. Six FFE fractions contained SDS-PAGE patterns, which were characteristic for the 20S proteasome (compare Fig. 1A with Fig. 2A). The pH of those fractions ranged from pH 4.91 to 5.27 (Fig. 1B). Fraction 36 contained the highest 20S concentration, indicating a pI of pH 5.06 for hepatic 20S complexes. Immunodetection of proteasome subunit α7 after western blotting of the fractions confirmed the presence of 20S in the fractions (Fig. 1C). Similar to the pattern analysis in Fig. 1A, subunit α7 (28.3 kDa calculated) could be detected in the fractions 34 to 38 by immunodetection (Fig. 1C). Furthermore, the detected amounts of subunit α7 matched the intensities of the bands in the pattern analysis, and thus confirmed fraction 36 as main proteasome fraction.

The reproducibility of IEF-FFE was monitored by performing multiple runs and measuring the pH in each fraction. A characteristic delineation of the measured pH gradient is depicted in Fig. 1B. According to the measured pH gradient, a difference as small as 0.09 pH units in average could be separated in the range from pH 3.51-9.43. In fact, the pH gradient was shallower in certain pH ranges, resulting in a higher resolution, for instance, in the range, where 20S proteasome focused. The performance during IEF-FFE in terms of laminar flow and reproducible formation of the pH gradient was monitored by adding highly soluble and visible pH markers with low molecular weight to the sample.

FFE sustains 20S structure and proteolytic activity during IEF
For downstream analyses after native IEF of multi-protein complexes, the structural integrity is essential. The presence of the characteristic 20S pattern after SDS-PAGE of the fractions by itself already indicates that the complex was separated rather than its subunits (Fig. 2A). Furthermore, single bands in a 1D lane at the molecular weight range between 20 and 40 kDa were not detected in any FFE fraction after native IEF of the 20S. The lack of such bands emphasizes that 20S subunits did not dissociate from the complex. Still, partial degradation of the complex during separation by FFE could be a possibility for the distribution of the 20S in more than one fraction as shown in Fig. 1A. Mapping of proteasome subunits in individual fractions obtained by native IEF-FFE of 20S shows a 2-DE pattern similar to those published previously (Fig. 2B; [6, 7]). Thus, 2-DE confirmed that all 20S subunits are present in FFE fractions containing 20S complexes shown in Fig. 1A. Finally, blue native electrophoresis of FFE fractions uniformly demonstrated the presence of a single protein band at similar molecular weight, demonstrating that 20S complexes were preserved in their unaltered, native quaternary structure (Fig. 2C). Proteasome activities in 20S fractions were also measured and are presented later in the results.

Development of novel FFE protocols for high resolution IEF of native protein complexes

The resolution in pH 3-10 is optimal for pI determination of protein complexes, but insufficient for the differentiation of small changes in complex assembly as hypothesized for 20S subpopulations. Therefore, protocols for high resolution IEF-FFE were developed. However, while improving the resolution, two major problems occurred. First, steady state IEF of 20S complexes could not be achieved by conventional FFE. Second, a white line, indicating protein precipitation, appeared during IEF. Thus, determination of the pIs of 20S complexes was initially imprecise and varied from the values determined in the gradient from pH 3-10 (Fig. 3A). The variation of the pI to approx. pH 5.4 in pH 4-6 indicated that the mobility of the complexes was too low to migrate towards its pI in narrow pH ranges, while remaining 20 min in the separation chamber. Therefore, increasing the time for IEF was mandatory. This was finally achieved by alteration of the FFE from continuous to interval mode. In the interval
mode, the media flow direction is repeatedly alternated between forward and backward after each interval period of 5 min. Therefore, running the FFE in interval mode enables that the sample remains in the separation chamber in a laminar flow for a hypothetically unlimited time period. For IEF of the 20S proteasome in pH ranges from 4-6, best results regarding accuracy and reproducibility were achieved by a separation period of one hour (Fig. 3B), as determined by increasing separation periods in 10 min steps up to 80 min (data not shown). Utilizing the optimized protocols, reproducibility for IEF-FFE of the native 20S complex could be demonstrated by separating different 20S preparations and starting with different protein concentrations. The separations shown in Fig. 2B and C both had the majority of protein complexes focusing at pH 5.05, which contained the characteristic 20S pattern and subunits as detected by immunoblotting. In repetitive separations of individual 20S purifications, approx. pH 5.05 could be confirmed to be the experimental pI of the predominant 20S form in murine liver. The average resolution achieved in the pH range from 4-6 was approximately 0.04 pH units, surpassing previously achieved resolution in 20S IEF [42, 43]. Like the pH gradient 3-10, the pH gradient 4-6 was actually shallower in the range where the 20S proteasome complexes focused, resulting in an even higher resolution than the average resolution (Fig. 4C).

20S complexes purified from heart have distinct pls from hepatic 20S

Since 20S inhibition caused ambiguous results in cardiovascular research, we were particularly interested whether proteasome heterogeneity exists in cardiac tissue. Therefore, 20S proteasome was purified from murine hearts to apparent homogeneity and subjected to the same protocols established for native IEF-FFE of liver 20S in the pH gradient from 4-6. After native IEF-FFE, the fractions were analyzed by SDS-PAGE for cardiac proteasome content in the same way as described for liver 20S (Fig. 4). Similar to the separation of native liver 20S, cardiac 20S was found in several fractions, but in contrast, cardiac 20S fractions ranged from pH 5.10 to 5.33 (Fig. 4B). Although the number of fractions containing cardiac 20S depended to a certain level on the total 20S amount applied to FFE (Fig. 4A and B), cardiac 20S focused in a distinct pH range from hepatic 20S (Fig. 3B). Interestingly, the pH of the fractions containing the highest
The concentration of cardiac 20S was around pH 5.26 as determined by repetitive IEF-FFE of different cardiac 20S preparations and at different concentrations. Thus, the predominant form of cardiac 20S proteasome is 0.2 pH units more alkaline than the predominant form in liver (pI 5.05). The depiction of the measured pH in fractions corresponding to those shown in Fig. 4A and B demonstrates the reproducibility of IEF-FFE in the pH range from 4-6 (Fig. 4C). Finally, it could be observed that cardiac 20S was separated from impurities (Fig. 4B), which remained after the multiple step purification as described in the experimental procedures. Thus, IEF-FFE facilitates further purification of 20S proteasome complexes.

**Different fractions contain 20S complexes with distinct pIs**

The distribution of 20S in different fractions shown in Figs. 1-4 could be interpreted as different forms of the 20S proteasome, which have different pIs, as well as imprecise focusing of the complex due to the low solubility at its pI. Whether the distribution derives from different 20S forms or poor focusing was determined by repeating IEF-FFE after pooling already separated FFE fractions. The major fraction containing the highest 20S proteasome concentration was not included in the pool to determine whether a similar distribution as in Figs. 1-4 would be produced or two major fractions would appear. The former would indicate a solubility problem at the pI of the complex and the latter different 20S forms with different pIs. A representative result of these experiments is shown in Fig. 5. FFE fractions C7 and E7 of separated cardiac 20S complexes were combined and again subjected to IEF-FFE. As expected from complexes having different pIs, the SDS-PAGE analysis of the second FFE shows a different distribution from those in Figs. 1-4. There is not one major fraction, but two (C7 and E7) with a fraction containing significantly lower 20S concentration in-between (D7; Fig. 5B). Furthermore, the pH values of the fractions containing the highest 20S concentration in the second separation match to the fractions of the first separation, which were combined for this experiment. Although more than two fractions in the second IEF-FFE contain 20S, the different distribution and the missing major fraction both indicate that 20S complexes with distinct pIs were separated in these experiments. The occurrence of 20S proteasomes in additional fractions might be explained by a slight shift in the pH.
gradient during the second IEF-FFE and by different splitting of the fractions at the fraction collector. Instability of the 20S forms due to loss of charge groups would be another explanation for having more fractions than expected in the second IEF-FFE.

**Proteasome complexes with distinct pls exhibit unique proteolytic patterns**

Proteolytic activities in individual fractions were compared to determine whether proteasome complexes with different pls have distinct activity. Since the proteasome has caspase- as well as trypsin- and chymotrypsin-like proteolytic activities, three different reporter peptides coupled to fluorophores were used to address each 20S activity individually. As expected from the structural analyses (Fig. 2A), all fractions containing 20S complexes degraded each of the three different reporter peptides (Fig. 6A), demonstrating full 20S functionality of complexes with different pls. However, quantitative comparison of the proteolytic activities across the fractions revealed remarkable differences between them. Although fractions D7 and F7 contain similar 20S concentrations (Fig. 6B), their proteolytic activities are significantly distinct. Trypsin-like activity in D7 is more than 40% higher than in F7. Interestingly, the proteolytic activities in D7 are not generally higher, since there is almost no change in chymotrypsin-like activity compared to F7. The ratios of proteolytic activities seem to be unique across 20S fractions. Therefore, proteasome complexes with different pls have distinct proteolytic activities and unique proteolytic patterns. Still, a general scheme for the proteolytic patterns across the fractions seems to be recognizable. The 20S complexes with rather acidic pls exhibit higher trypsin-like than caspase- and chymotrypsin-like activities. Towards the alkaline end, the proteolytic pattern is the opposite way around and the trypsin-like activity is generally lower than the other two activities. This result might suggest a pH dependency of 20S proteolytic activities. However, influence of the pH in the separation medium can be excluded, since the small differences in pH across the fractions were compensated by the assay buffer and all proteolytic assays were conducted at pH 7.5.

For further verification of distinct proteolytic activities across the fractions, the proteolytic assays were extended to include different substrate to proteasome ratios and exclude the possibility of substrate dependency of the observed difference. Therefore, the
activities in fractions D7 and F7 were assayed at substrate concentrations of 25, 50, 100, 200 and 500µMol (Fig. 7). The differences in proteolytic activities between fractions D7 and F7 as observed in Fig. 6A were reproducible at all substrate concentrations and more pronounced with increasing concentration. For instance, at 500µMol the trypsin-like activity in D7 was 64% higher than in F7 compared to a difference of 43% at 100µMol substrate concentration (data not shown). The caspase-like activity was also significantly higher in D7 than in F7 (+32%) at 500µMol substrate concentration. In contrast, the chymotrypsin-like activity exhibited no significant difference between D7 and F7 at any substrate concentration. Therefore, measuring the proteolytic activities at higher and lower substrate concentrations validated the results shown in Fig. 6A, where 100µMol substrates were used. For comparison of the unique proteolytic pattern of 20S complexes in fractions D7 and F7, the results obtained from various substrate concentrations were used to calculate ratios of proteolytic activities (Fig. 7). The curves in the plots are almost parallel, emphasizing a linear proportionality of activity ratios independent of substrate concentrations. At all analyzed substrate concentrations, the ratio of caspase- to trypsin-like activities in F7 was between 22-33% higher than in D7 (two tailed, paired T-Test p<0.005; Fig. 7A). Chymotrypsin- to trypsin-like activity ratios follow a similar proportionality and are in average 45±4.6% higher in F7 (p=0.01; Fig. 7C). In contrast, the ratio of caspase- and chymotrypsin-like activities exhibited no significant difference between the two fractions (Fig. 7B). Thus, the results obtained at higher substrate concentrations emphasize those showing that the proteolytic activities of distinct complexes are uniquely combined (Fig. 6), which in turn suggests individual incorporation of inducible subunits or modulation of the activities by PTMs.

**Unique 20S subunit compositions across 20S IEF-FFE fractions**

The results presented in Figs. 6 and 7 demonstrated that 20S complexes separated by FFE exhibited distinct proteolytic patterns. Substrate specificity as well as turnover of the 20S is dependent on incorporation of constitutive and inducible β1, β2 and β5 subunits, since they bear the proteolytic sites. To address whether differences in 20S assembly contribute to the distinct proteolytic patterns observed in separated 20S fractions, the concentrations of constitutive and inducible β1, β2 and β5 subunits were
analyzed by Western Blotting (Fig. 8). For comparability with Figs. 6 and 7, particular focus in Fig. 8 was kept on fractions D7 and F7. Comparing the amount of proteasome subunits in D7 and F7, all analyzed subunits except β1i and β2i were detected at a similar level (Fig. 8B). Interestingly, β1i and β2i expressions are neither exhibiting a similar trend nor are all inducible β subunits changing in unison. While β1i levels are increased by 47% in D7 (p<0.05), β2i is reduced by 58% in F7 (p<0.01; Fig. 6B). This result is unexpected, since current 20S models suggest incorporation of either constitutive or inducible subunits. Furthermore, reduced levels of constitutive subunits would be expected with increasing amounts of the corresponding inducible subunits and vice versa. However, immunodetection of the inducible subunits in cardiac samples is quite challenging, since they occur at much lower amounts than the constitutive subunits (approximately 1-5%). Therefore, a subunit exchange occurs at the quantitative level of 1:1, but is not detectable at the same ratio in Western Blots. Individual variation of inducible subunit levels across the separated 20S complexes suggests the presence of intermediate 20S proteasomes with mixed assembly of constitutive and inducible subunits. Still, the predominant 20S assembly in cardiac tissue is composed of constitutive subunits. In summary, the results demonstrate that 20S complexes separated by IEF-FFE have unique compositions, which might contribute to their distinct proteolytic activities.

**Different levels of 20S phosphorylation are distinguishable by IEF-FFE**

Alternate molecular compositions or PTMs could be the origins for the minor variance of 0.2 pH units in pls between the majority of cardiac and hepatic proteasomes. Since proteasomes purified from both organs contain all known 20S subunits, no additional associated proteins were detected, and the size of all proteasomes is similar, variations in molecular compositions are unlikely. In contrast, different amounts of additional charge groups, such as phosphorylations, remain easily undetected in our analysis and potentially influence the pl significantly. Several 20S subunits have been reported to be phosphorylated, some of them even at multiple sites [26, 44]. Furthermore, phosphorylation seems to modulate 20S proteasome activity [6, 45]. Liver 20S complexes are more acidic than cardiac 20S and possibly higher phosphorylated.
Therefore, 20S proteasome complexes that are phosphorylated to different extents were produced \textit{in vitro} to assess their impact on the pI of 20S in native IEF-FFE. The modification of 20S was derived by partial enzymatic dephosphorylation with CIAP. As expected, the pIs shifted after partial dephosphorylation of 20S to the alkaline range (Fig. 9). In total, 20 FFE fractions ranging from pH 5.0 (F53) to 5.58 (F72) contained the characteristic 20S SDS-PAGE pattern. Remarkably, five major fractions appeared in native IEF-FFE at pH 5.13, 5.19, 5.24, 5.35 and 5.54. Each major fraction having higher protein amounts than the neighboring fractions as measured by the intensities of all bands in a lane (Fig. 9B). These fractions probably contain the most stable phosphorylation forms produced by the partial dephosphorylation. The occurrence of five different major fractions could be explained by sequentially losing at least one highly exposed phosphor group with favorable access for CIAP from acidic to alkaline pIs. Since the pI of one dominant form at pH 5.24 matches the pI of the predominant cardiac 20S complexes, the slight difference in pIs indeed could be explained by a difference in phosphorylation state. Finally, this experiment confirms the hypothesis that every fraction contains a physically different form of the 20S proteasome. Whether the differences result from major conformational changes, posttranslational modifications, or different structural assembly remains to be explored.
Discussion:

The present study is the very first to document the existence of multiple proteasome subpopulations in the heart. Essentially, the analyses were enabled by the development of novel protocols for in-solution IEF of multi-protein complexes in a laminar flow. The final protocol for pH 4-6 supported an average resolution of 0.04 pH units and was highly effective to differentiate 20S proteasome complexes purified from murine heart and liver. With the described developments, cardiac proteasomes are easily distinguishable from liver proteasomes by their difference in pls of 0.2 pH units. Furthermore, cardiac as well as hepatic 20S complexes were separated into multiple subpopulations, which have distinct proteolytic activities and molecular compositions. Partial de-phosphorylation and separation of native liver 20S proteasomes indicated that the difference in the pls of the dominant 20S form in heart and liver might result from alternative extent of protein phosphorylation.

The technology employed in our study is a well established technology for separation of peptides, proteins and organelles [34-36]. However, protocols for the separation of protein complexes were lacking so far. The presented data demonstrates that FFE facilitates further purification and differentiation of 20S complexes after elaborate biochemical purification, ending with ion exchange chromatography. Furthermore, fractionation by FFE resulted in highly reproducible separations of native 20S complexes, indicating 20S heterogeneity at quaternary structural level.

Previous investigations on 20S complexes from various organisms and tissues, report pls ranging from 4.6 in yeast to 6.67 in ostrich skeletal muscle [46, 47]. The pls for 20S isolated from cardiac and hepatic murine tissues determined in the present study are very similar to the pl of 5.1-5.2 documented for rat proteasome isolated from skeletal muscle [42]. The resolution for IEF in the study on rat 20S was approximately 0.15 pH units (deduced from the published figures). Other reports published the pl at lower resolution of approximately 0.5 pH units [43]. Since the developed protocols facilitated separation of minute differences in pls at a resolution of 0.04 pH units in average, the
present manuscript provides the first report for high resolution IEF based separation of proteasome complexes.

Several reports indicate the co-existence of constitutive and immunoproteasomes in homogeneous cell populations and single tissues without previous stimulation [26]. Mostly, these studies demonstrate the presence of both constitutive and inducible β subunits in 20S purifications by a combinatorial approach of 2-DE and MS (reviewed in [48]). Furthermore, up to 32 spots on 2-DE gels from 20S purifications were identified as one of 17 different proteasome subunits, indicating PTMs [26]. Several PTMs of proteasome subunits, such as acetylation or phosphorylation, were already identified and are increasingly discovered mainly due to advancements in mass spectrometry [48]. Although those and other reports indicate the existence of proteasome heterogeneity, only separation and isolation of different proteasome complexes provide proof for heterogeneity at native protein level. To our knowledge, one single group demonstrated the presence of 20S subtypes in single tissues so far [8, 9].

First indication for additional 20S subtypes besides the constitutive and immunoproteasomes was found in rat skeletal muscle [8]. In detail, high resolution ion-exchange chromatography of purified 20S complexes resulted in overlapping, but distinct peaks in chromatograms [9]. Peaks in chromatograms of 20S separations obtained from spleen, liver and kidney were closely overlapping and less pronounced [8]. Skeletal muscle derived 20S complexes, represented by the individual peaks, contained different amounts of inducible subunits and had different proteolytic activities. In total, six proteasome subtypes for rat skeletal muscle were suggested, which potentially have different cellular functions or present complexes in different regulatory states. Quantitative alterations of 20S subtypes have been demonstrated in a disease related context by studying diabetic rats [49]. However, even by employing advanced proteomic technologies, the nature of the differences in subtypes remained obscure [29]. Targeting of all 20S subtypes with a single inhibitor in diseases where protein degradation is disturbed, might result in a positive net effect, but is likely accompanied by preventable side effects. Dahlmann et al. state in their manuscript that current 20S
preparations and analyses probably all contain mixtures of 20S subtypes [8]. For future evaluation of 20S proteasomes as therapeutic targets, the characterization of subtypes and description of their function seems to be crucial.

The 20S subtypes presented in this study are distinguishable by their proteolytic activities and composition of β subunits, which is similar to reported differences of subtypes in rat skeletal muscle [8]. In contrast, constitutive proteasomes seemed to elute earlier than their immuno-counterparts when separated by high resolution chromatography. The proteasome subtypes eluting in-between were suggested to be intermediate proteasomes, containing a mixture of the constitutive and inducible subunits. Separation by pl seemed not to distinguish between constitutive and immunoproteasome, but the presence of intermediate forms is indicated by individually in- or decreased incorporation of β subunits. Therefore, our results support the hypothesis of existing intermediate proteasomes. The pls of inducible subunits except β5i differ minimally from their constitutive forms and their influence on the pls of complexes is presumably minute. In contrast, PTMs are likely to influence the pls of 20S complexes, since i) cardiac and hepatic proteasomes both contain all known α and β subunits, but their pls are significantly different, ii) hepatic 20S complexes are distributed over a broader pH range, and iii) partially dephosphorylation of liver 20S results in complexes with pls encompassing more than 0.5 pH units. Unknown interacting partners might also influence the pls of 20S complexes. However, the sizes of cardiac or hepatic 20S as well as subpopulations appeared similar on native gels.

Phosphorylation of 20S subunits seems to regulate multiple mechanisms associated with proteasome structure and activity [48]. For instance, phosphorylation modulates the proteolytic activity of cardiac 20S [6], and α7 phosphorylation is associated with 26S stabilization [8]. In the present study, the extent of 20S phosphorylation has a tremendous effect on its pl, altering it up to 0.5 pH units. Furthermore, after partial dephosphorylation of liver 20S, particular dephosphorylated complexes appeared at similar pls and distribution as cardiac 20S complexes. Therefore, it is conceivable that cardiac and hepatic 20S differ in their extent of phosphorylation. Supporting in this
matter is that proteasome activity in liver is significantly higher than in heart, and phosphorylation of cardiac 20S promotes its activity [6]. It will be further interesting to analyze, which subunits were dephosphorylated in our experiments and how this affects proteasome activity.

Distinct proteasome activities of subpopulations were measurable for heart as well as for skeletal muscle derived 20S (present study and [8, 9]). Interestingly, in both studies a common trend in the proteolytic patterns of the subpopulations is apparent. When the percentages of caspase- and chymotrypsin-like activities were relatively high for a subpopulation, the trypsin-like activity was relatively low and vice versa. This observation is mostly remarkable, because proteasome composition of constitutive and inducible β subunits across subpopulations followed different patterns in the studies. Furthermore, variable amounts of incorporated constitutive and inducible β subunits would be expected to have an opposite effect on caspase- and chymotrypsin-like activities, since subunit β1 changes from caspase- to chymotrypsin-like activity when exchanged by its inducible counterpart [32, 33]. Therefore, substrate specificity and turnover by subpopulations seem not to be determined singly by proteasome assembly. Meanwhile, it was revealed that interferon-gamma induces altered levels of proteasome phosphorylation besides proteasome assembly [44, 45]. Thus, PTMs might influence proteasome function to a higher extent than recognized by our current knowledge and models.

In conclusion, the application of a novel protocol for in-solution IEF of multi-protein complexes enabled the separation of distinct native proteasome complexes. Cardiac and hepatic proteasomes displayed unique pIs defined by distinct molecular compositions, proteolytic activities, and substrate preference. In addition, subpopulations may also be contributed by possible differential posttranslational modification of the proteasome subunits. These data provide mechanistic insights for selective modulation of proteasome complexes in the heart. The current inhibitors abolish proteasome activities by affecting all 20S proteasome populations [1, 50, 51].
The possibility to distinguish 20S proteasomes derived from different tissues as well as within a single organ provides the potential of more accurate therapeutic targeting.
Acknowledgements:
This study was supported in part by the AHA Fellowship Award (0625062Y to Dr. Oliver Drews), and by the proteomic core of NHLBI program project (P01-080111 to Dr. Peipei Ping).
References:


Figure Legends:

**Fig. 1:** A) Purified 20S proteasome from murine liver separated by FFE under native conditions according to the pl in a pH gradient from pH 3-10. Equal amounts of each fraction were subsequently separated by SDS-PAGE and visualized by silver staining. Indicated numbers correspond to FFE fractions with the pH indicated. Approximately 0.6 μg purified 20S proteasome diluted in FFE buffer were separated in lanes marked with S. B) Linearity of the pH gradient in FFE pH 3-10 determined by pH measurements of each fraction. Indicated are the fractions, which were applied on SDS-PAGE (lines) and contained 20S proteasome (arrows down) as well as the main fraction containing 20S (arrow up). C) Immunodetection of proteasome subunit α7 in fractions after native IEF-FFE of purified liver 20S. Equal amounts of indicated fractions were subjected to SDS-PAGE and subsequently immunoblotted. Fractions correspond to those shown in A. S: approx. 1.3 μg purified 20S proteasome diluted in FFE buffer; C: approx. 1μg purified 20S proteasome.

**Fig. 2:** Structural analyses of proteasome fractions demonstrate all 20S subunits are present in one complex after native IEF-FFE. A) SDS-PAGE of a proteasome fraction shows 9 bands, which were identified by immunodetection in parallel experiments; SYPRO Ruby staining. B) 2-DE of proteasome fractions after native IEF-FFE displays the characteristic 20S subunit pattern as previously published [6, 7]; silver staining. C) Native electrophoresis of proteasome fractions after fractionation by FFE in a pH gradient from 4-6 resulted in a single high molecular weight band for every fraction. Equal volumes of fractions were separated by blue native electrophoresis and subsequently Coomassie stained. Representative results are shown.

**Fig. 3:** Comparison of native IEF-FFE in the pH gradient 4-6 of purified 20S proteasome from murine liver. A) Fractions obtained by operating the FFE in conventional continuous mode. B) and C) Separation of fractions after operating the FFE in interval mode for 1h. Indicated numbers correspond to FFE fractions and their pH values, S represents 20S diluted with FFE buffer. In A) and B), approximately 60 μg 20S was
separated by FFE, while 10 μg were used in C), demonstrating reproducible IEF in interval mode at different protein concentrations.

**Fig. 4:** 20S proteasome separated by native IEF-FFE in pH 4-6 after purification from murine heart. FFE fractions containing cardiac proteasome complexes were separated by SDS-PAGE and the corresponding pH values determined. A) 60 μg or B) 20 μg of purified cardiac 20S proteasome were separated by FFE. Equal amounts of fractions were subjected to SDS-PAGE and subsequently silver stained (S: unfractionated sample). C) pH values of each fraction shown in A) ▲ and B) □.

**Fig. 5:** FFE fractions of purified 20S reproducibly focus at similar pH values when separated a second time by FFE. A) SDS-PAGE of fractions after native IEF-FFE of 60 μg cardiac 20S complexes in pH 4-6. B) SDS-PAGE of separated cardiac 20S complexes after pooling fractions C7 and E7 shown in A and repeated native fractionation by IEF-FFE in pH 4-6. In both cases, FFE was performed in interval mode. Quantification of 20S bands was performed after SYPRO Ruby staining. Indicated is the percentage of the fraction with the highest 20S concentration.

**Fig. 6:** Proteasome complexes with distinct pIs differ in their proteolytic activities. A) After separation of cardiac 20S complexes by interval IEF-FFE in pH 4-6, the caspase-, trypsin- and chymotrypsin-like activities were assayed in triplicates. Displayed are mean values with their corresponding standard errors. Since the peptide for chymotrypsin-like activity has a much higher turnover than the other two peptides, the percentage of the highest activity, found in E7, is indicated. A direct comparison of D7 and F7, which contain the similar proteasome concentrations, is shown in the right diagram. B) Quantification of proteasome complexes in fractions corresponding to those shown in A by SYPRO Ruby staining. Concentrations are indicated as percentage of the fraction with the highest proteasome content.

**Fig. 7:** The proteolytic activities of proteasomes with distinct pIs are reproducibly distinguishable at different substrate concentrations. Fractions D7 and F7 from native
interval IEF-FFE of cardiac 20S were assayed at substrate concentrations from 25-500 µMol (n=3). Subsequently, the ratios of caspase- to trypsin- (A), caspase- to chymotrypsin- (B), and chymotrypsin- to trypsin-like (C) activities at all corresponding concentrations were calculated for D7 and F7 and plotted (mean values and standard errors). By comparison of the ratios in D7 to F7, an almost linear proportionality at each substrate concentration is visible. The distance of this proportionality was tested with a paired, two tailed T-Test (p values) and confident for the distance of ratios shown in A and C.

**Fig. 8:** Differences in molecular composition of proteasome complexes with distinct pIs. Fractions D7 and F7 were obtained by native interval IEF-FFE of cardiac 20S proteasomes and subsequently immunoblotted for the presence of constitutive (α3, β1, β2 and β5) and inducible 20S subunits (β1i, β2i and β5i). The fractions correspond to those with distinct proteolytic signatures shown in Fig. 7. A) Equal protein amounts of D7 and F7 were immunoblotted in triplicates. B) Intensities of bands from Western Blots shown in A were quantified and displayed as average percentage of corresponding signals in F7 (n=3). Standard errors are displayed. Quantities of β1i and β2i were significantly different at p<0.05 (*) and p<0.01 (**) respectively.

**Fig. 9:** Different phosphorylated forms of hepatic 20S complexes separated by native interval IEF-FFE in pH 4-6. Prior to FFE, 20S was partially dephosphorylated by CIAP. Equal amounts of FFE fractions were separated by SDS-PAGE and silver stained. The intensities of all protein bands in the lanes were measured by the Scion Image software and indicated in the graph. Five fractions contained higher protein concentrations than neighboring fractions, probably containing distinctly phosphorylated proteasome complexes. These fractions focused at pH 5.13, 5.19, 5.24, 5.35 and 5.54.
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