Mammalian Proteasome Subpopulations with Distinct Molecular Compositions and Proteolytic Activities*

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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 have 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 multiprotein complexes in a laminar flow that 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 to 5.33. In contrast, the majority of hepatic 20 S proteasomes had a pI of 5.05 and focused from pH 5.01 to 5.29. Importantly proteasome subpopulations degraded specific model peptides with different turnover rates. Among cardiac subpopulations, proteasomes with an approximate pI of 5.21 showed 40% higher trypsin-like activity than those with pI 5.28. Distinct proteasome assembly may be a contributing factor to variations in proteolytic activities because proteasomes with pI 5.21 contained 58% less of the inducible subunit β2i compared with those with pI 5.28. In addition, dephosphorylation of 20 S proteasomes demonstrated that besides molecular composition posttranslational modifications largely contribute to their pI values. These data suggest the possibility of mixed 20 S 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. Molecular & Cellular Proteomics 6:2021–2031, 2007.

The half-life of many proteins in cell proliferation, apoptosis, and gene expression is dependent on the ubiquitin-proteasome system (UPS)1 (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), raising 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 26 S proteasomes recognize, unfold, and finally degrade polyubiquitinated proteins into short peptides (12–14). The degradation is catalyzed by the 20 S core complexes, which associate with one or two of the activating 19 S, 11 S, and PA200 complexes (15, 16). The proteolytic activities of the 20 S proteasomes are described as caspase-, trypsin-, and chymotrypsin-like (17, 18). The proteolytic active sites are located on the proteasome subunits β1, β2, and β5 (19–21). In total, 14 α and 14 β subunits constitute 20 S proteasomes, forming four stacked rings with seven unique α subunits in the two outer rings and seven unique β subunits in the two inner rings (22, 23). A second subpopulation with distinct proteolytic activities is assembled when β1, β2, and β5 are substituted by β1i, β2i, and β5i upon induction by interferon-γ in

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mammals (24, 25). Remarkably co-expression of inducible β 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 20 S proteasome complexes: the constitutive and the immuno forms. Additional 20 S subtypes were suggested by Dahlmann et al. (8, 9), but further evidence is imperative to establish their existence in universally effective UPS models.

Proteomics technologies have been essential in characterizing the UPS in the past (6, 7, 26–30). However, it is hitherto unknown in what manner 20 S subunits can be assembled besides the constitutive and the immuno forms. Analytical high resolution chromatography of native 20 S proteasomes from rat skeletal muscle suggested the existence of intermediate 20 S complexes composed of both constitutive and inducible β subunits (8, 9). The molecular compositions of 20 S proteasomes have been recognized to play a pivotal role in their proteolytic activities (24, 25, 31). Individual altered expression of subunits bearing the proteolytic sites significantly impacts the 20 S 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 20 S 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 group and other groups indicated charge differences between subpopulations by different assembly and posttranslational modifications (PTMs). Therefore, separation according to the pl appeared to be essential. Native IEF at high resolution of multiprotein complexes in excess of 700 kDa, such as the 20 S proteasomes, 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 a solid matrix (34–36). In our study, a novel FFE protocol was used for IEF to effectively separate 20 S proteasome complexes.

**EXPERIMENTAL PROCEDURES**

**Purification of 20 S 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 Applied Science), and phosphatase inhibitor mixtures 1 and 2 (Sigma) and centrifuged at 25,000 × g for 2 h. Next ammonium sulfate was added slowly to the supernatant at a final concentration of 40%, and then the sample was centrifuged at 12,000 × g for 1 h. The procedure was repeated with the supernatant to achieve a final concentration of 60% ammonium sulfate, and again the sample was centrifuged at 12,000 × g for 1 h. The pellet was dissolved in 20 mM Tris (pH 7.4), 5 mM MgCl₂, and 1 mM DTT and dialyzed against the same solution overnight. Then the dialyzed sample was reconstituted to 45% B (A: 20 mM Tris (pH 7.4), 5 mM MgCl₂, 0.5 mM DTT, and 10% glycerol; B: same as A plus 600 mM KCl) and loaded on an XK 26 40 column (GE Healthcare) packed with Q Sepharose (GE Healthcare), which was equilibrated with 45% B. After washing the column, B was increased to 75% and the eluant was collected and centrifuged at 205,000 × g for 19 h. The pellet was dissolved by homogenization in A and centrifuged at 3,000 × g to remove insoluble particles. Then the supernatant was loaded on a Mono Q 5/50 column (GE Healthcare) equilibrated with A. Finally purified 20 S proteasomes were eluted at ~55% B by running a shallow gradient up to 100% B. All steps were performed at 4 °C or on ice.

**Dephosphorylation of 20 S Proteasomes**—Partial dephosphorylation of 20 S proteasomes complexes was achieved by calf intestinal alkaline phosphatase (CIAP, Promega, Madison, WI) treatment. In variation with the 10× buffer supplied by the provider, dephosphorylation was performed in buffer lacking spermidine, thus containing 0.5 mM Tris (pH 9.3), 10 mM MgCl₂, and 1 mM ZnCl₂. In total, 60 μg of purified 20 S proteasomes were incubated in the presence of 250 units of CIAP for 30 min at 37 °C.

**Isoelectric Focusing Using Free Flow Electrophoresis**—Free flow electrophoresis was performed on the BD FFE System (BD Biosciences). For high resolution FFE, a 0.4-mm spacer was used, resulting in a total volume of 20 ml in the separation chamber. Accurate and reproducible determination of the isoelectric point of the 20 S 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 seven inlets for the separation and stabilizing 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 medium containing 1% Serva-lytes 4–6 and 0.3% Serva-lytes 3–10. All media used for separation, stabilization as well as counterflow, contained 0.2% (hydroxypropyl)-methyl cellulose (average molecular weight 86,000, Aldrich) and 25% glycerol. In addition, 1 mM DTT was added to all media. Anodic and cathodic electrode solutions were prepared according to the manufacturer and contained 100 mM H₂SO₄ and 100 mM NaOH, respectively.

Samples subjected to FFE were diluted 1:10 with separation medium. In general, 200 μl of diluted sample was applied containing typically 60 μg of purified 20 S proteasome complexes. For visualization of injection, separation, and fractionation, 1.5 μl of sulfanilic acid azochromotrop (1% (w/v), Sigma) was added to each sample. The diluted samples were injected into the separation chamber at the cathodic sample inlet (S4) using flow rates of 1 ml/h for continuous and 9 ml/h for interval mode with the improved separation protocol. Before and after sample entry, undiluted separation medium was injected into 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 60 ml/h, and IEF conditions were limited to 1500 V and 50 mA. For high resolution separation in narrow pH gradients, the media flow rate during sample entry was 300 ml/h. Then prolonged IEF was performed at a maximal 1200 V and 30 mA in interval mode with
intervals set to 5 min and a reduced flow rate of 50 ml/h. Operating the FFE can be visualized as virtual elongation of the separation chamber and thus extension of 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 20 S complexes was achieved after 1 h 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 and the interval modes, fractions were collected in 96-well plates when the red marker sulfanilic acid azochromotrop 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, 18-cm IPG strips (GE Healthcare) according to a modified protocol of Gorg et al. (38). The IPG strips were rehydrated in 350 μl of buffer containing 8 M (w/v) urea, 1% (w/v) CHAPS, 0.2% (w/v) DTT, and 0.5% (v/v) Pharmalytes 3–10. Prior to IEF, FFE fractions were diluted at a ratio of 1:5 with buffer containing 9 M (w/v) urea, 4% (w/v) CHAPS, 2% (w/v) DTT, and 1% (v/v) Pharmalytes 3–10. Sample entry was facilitated by 30 min at 150 V, 30 min at 300 V, and 30 min at 600 V. After that, the voltage was increased to 8000 V using a 30-min voltage gradient, and IEF reached a steady state after a minimum of 18 kV-h. The second dimension was performed on 12% acrylamide gels after equilibration of the samples as published previously (39). Finally the separated proteins were visualized by silver staining.

For one-dimensional SDS-PAGE, FFE fractions 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 blue native sample buffer and separated on 6% acrylamide gels. Ferritin complexes (molecular mass, 440 kDa) and thyroglobulin (molecular mass, 669 kDa; GE Healthcare) were used as molecular weight standards.

Immunodetection of 20 S 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, and used at dilutions from 1:1500 to 1:2000. Horseradish peroxidase-conjugated goat anti-mouse (Sigma) and goat anti-rabbit (BD Biosciences) were used as secondary antibodies at a dilution of 1:1500 to 1:2000. Horse- 

Native IEF of 20 S Proteasome Complexes Purified from Mouse Liver—Because the size of the native 20 S proteasome exceeds 700 kDa, FFE was chosen for native IEF of multiprotein 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 20 S proteasome complexes in the pH range from 3 to 10 (Fig. 1). Finally the fractions were mixed with SDS buffer and separated by SDS-PAGE. Six FFE fractions contained SDS-PAGE patterns, which were characteristic for the 20 S proteasomes (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 concentration of 20 S proteasomes, indicating a pl of pH 5.06 for hepatic 20 S complexes. Immunodetection of proteasome subunit α7 after Western blotting of the fractions confirmed the presence of 20 S proteasomes in the fractions (Fig. 1C). Similar to the pattern analysis in Fig. 1A, subunit α7 (28.3 kDa, calculated) could be detected in fractions 34–38 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 the 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 on average could be separated in the range from pH 3.51 to 9.43. In fact, the pH gradient was shallow in certain pH ranges, resulting in a higher resolution, for instance, in the range where 20 S proteasomes 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 20 S Proteasome Structure and Proteolytic Activities During IEF—For downstream analyses after native IEF of multiprotein complexes, the structural integrity is essential. The presence of the characteristic 20 S proteasome 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 one-dimensional lane at the molecular mass range between 20 and 40 kDa were not detected in any FFE fraction after native IEF of the 20 S proteasomes. The lack of such bands emphasizes that 20 S 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 20 S proteasomes in more than one fraction as shown in Fig. 1A. Mapping of proteasome subunits in individual fractions obtained by native IEF-FFE of 20 S proteasomes shows a 2-DE pattern similar to those published previously (Fig. 2B and Refs. 6 and 7). Thus, 2-DE confirmed that all 20 S subunits are present in FFE fractions containing 20 S 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 20 S complexes were preserved in their unaltered, native quaternary structure (Fig. 2C). Proteasome activities in 20 S fractions were also measured and are presented later in "Results."

**Development of Novel FFE Protocols for High Resolution IEF of Native Protein Complexes**—The resolution in pH 3–10 is optimal for pl determination of protein complexes but insufficient for the differentiation of small changes in complex assembly as hypothesized for 20 S subpopulations. Therefore, protocols for high resolution IEF-FFE were developed. However, while improving protocols to increase the resolution, two major problems occurred. First, steady state IEF of 20 S complexes could not be achieved by conventional FFE. Second, a white line, indicating protein precipitation, appeared during IEF. Thus, determination of the pl values of 20 S complexes was initially imprecise and varied from the values determined in the gradient from pH 3 to 10 (Fig. 3A). The variation of the major pl to approximately pH 5.4 in pH 4–6 indicated that the mobility of the complexes was too low to migrate toward their pl values 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 the sample to remain in the separation chamber in a laminar flow for a hypothetically unlimited time period. For IEF of the 20 S proteasomes in pH ranges from 4 to 6, best results regarding accuracy and reproducibility were achieved by a separation period of 1 h (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 20 S complex could be demonstrated by separating different 20 S preparations and starting with different protein concentrations. The separations shown in Fig. 2, B and C, both had the majority of protein complexes focusing at pH 5.05, which contained the characteristic 20 S proteasome pattern and subunits as detected by immunoblotting. In repetitive separations of individual 20 S proteasome purifications, approximately pH 5.05 could be confirmed to be the experimental pI of the predominant 20 S form in murine liver. The average resolution achieved in the pH range from 4 to 6 was $10^{-4}$ pH units, surpassing previously achieved resolution in IEF of 20 S proteasomes (42, 43). Like the pH gradient 3–10, the pH gradient 4–6 was actually shallower in the range where the 20 S proteasome complexes focused, resulting in an even higher resolution than the average resolution (Fig. 4).

The distribution of 20 S proteasomes in different fractions shown in Figs. 1–4 could be interpreted as different forms of the 20 S proteasome that have different pI values as well as imprecise focusing of the complex due to the low solubility at its pI. Whether the distribution is derived from

**Fig. 3.** Comparison of native IEF-FFE in the pH gradient 4–6 of purified 20 S proteasomes 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 1 h. The indicated numbers correspond to FFE fractions and their pH values; S represents 20 S proteasomes diluted with FFE buffer. In A and B, ~60 μg of 20 S proteasomes was separated by FFE, whereas 10 μg was used in C, demonstrating reproducible IEF in interval mode at different protein concentrations.

**Fig. 4.** 20 S proteasomes 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 were determined. 20 μg (A) or 60 μg (B) of purified cardiac 20 S proteasomes was 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 (□).

20 S Complexes Purified from Heart Have Distinct pI Values from Hepatic 20 S Proteasomes—Because inhibition of 20 S proteasomes caused ambiguous results in cardiovascular research, we were particularly interested in whether proteasome heterogeneity exists in cardiac tissue. Therefore, 20 S proteasomes were purified from murine hearts to apparent homogeneity and subjected to the same protocols established for native IEF-FFE of liver 20 S proteasomes in the pH gradient from 4 to 6. After native IEF-FFE, the fractions were analyzed by SDS-PAGE for cardiac proteasome content in the same way as described for liver 20 S proteasomes (Fig. 4). Similar to the separation of native liver 20 S proteasomes, cardiac 20 S proteasomes were found in several fractions, but in contrast, cardiac 20 S proteasome fractions ranged from pH 5.10 to 5.33 (Fig. 4B). Although the number of fractions containing cardiac 20 S proteasomes depended to a certain level on the total amount of 20 S proteasomes applied to FFE (Fig. 4, A and B), cardiac 20 S proteasomes focused in a distinct pH range from hepatic 20 S proteasomes (Fig. 3B). Interestingly the pH of the fractions containing the highest concentration of cardiac 20 S proteasomes was around pH 5.26 as determined by repetitive IEF-FFE of different cardiac 20 S proteasome preparations and at different concentrations. Thus, the predominant form of cardiac 20 S proteasomes 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. 4, A and B, demonstrates the reproducibility of IEF-FFE in the pH range from 4 to 6 (Fig. 4C). Finally it could be observed that cardiac 20 S proteasomes were separated from impurities (Fig. 4B), which remained after the multiple step purification as described under "Experimental Procedures." Thus, IEF-FFE facilitates further purification of 20 S proteasome complexes.

Different Fractions Contain 20 S Complexes with Distinct pI Values—The distribution of 20 S proteasomes in different fractions shown in Figs. 1–4 could be interpreted as different forms of the 20 S proteasome that have different pI values as well as imprecise focusing of the complex due to the low solubility at its pI. Whether the distribution is derived from
different 20 S forms or poor focusing was determined by repeating IEF-FFE after pooling already separated FFE fractions. The major fraction containing the highest 20 S proteasome concentration was not included in the pool to determine whether a distribution similar to that 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 would indicate different 20 S forms with different pI values. A representative result of these experiments is shown in Fig. 5. FFE fractions C7 and E7 of separated cardiac 20 S complexes were combined and again subjected to IEF-FFE. As expected from complexes having different pI values, the SDS-PAGE analysis of the second FFE showed a different distribution from those in Figs. 1–4. There is not one major fraction, but two (C7 and E7) with a fraction containing a significantly lower concentration of 20 S proteasomes in between (D7; Fig. 5B). Furthermore the pH values of the fractions containing the highest concentrations of 20 S proteasomes in the second separation matched those of the fractions of the first separation that were combined for this experiment. Although more than two fractions in the second IEF-FFE contain 20 S proteasomes, the different distribution and the missing major fraction both indicate that 20 S complexes with distinct pI values were separated in these experiments. The occurrence of 20 S 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 20 S 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 pI Values Exhibit Unique Proteolytic Patterns—Proteolytic activities in individual fractions were compared to determine whether proteasome complexes with different pI values have distinct activity. Because proteasomes have caspase- as well as trypsin- and chymotrypsin-like activities, three different reporter peptides coupled to fluorophores were used to address each 20 S proteasome activity individually. As expected from the structural analyses (Fig. 2A), all fractions containing 20 S complexes degraded each of the three different reporter peptides (Fig. 6A), demonstrating full 20 S proteasome functionality of complexes with different pI values. However, quantitative comparison of the proteolytic activities across the fractions revealed remarkable differences between them. Although fractions D7 and F7 contained similar concentrations...
of 20 S proteasomes (Fig. 6B), their proteolytic activities were significantly distinct. Trypsin-like activity in D7 was more than 40% higher than in F7. Interestingly, the proteolytic activities in D7 were not generally higher because there was almost no change in chymotrypsin-like activity compared with F7. The ratios of proteolytic activities seemed to be unique across 20 S proteasome fractions. Therefore, proteasome complexes with different pI values have distinct proteolytic activities and unique proteolytic patterns. Still a general scheme for the proteolytic patterns across the fractions seems to be recognizable. The 20 S complexes with rather acidic pI values exhibit higher trypsin-like than caspase- and chymotrypsin-like activities. Toward the alkaline end, the proteolytic pattern is the opposite, and the trypsin-like activity is generally lower than the other two activities. This result might suggest a pH dependence of 20 S proteolytic activities. However, influence of the pH in the separation medium can be excluded because 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 dependence of the observed difference. Therefore, the activities in fractions D7 and F7 were assayed at substrate concentrations of 25, 50, 100, 200, and 500 μM (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 500 μM the trypsin-like activity in D7 was 64% higher than in F7 compared with a difference of 43% at 100 μM substrate concentration (data not shown). The caspase-like activity was also significantly higher in D7 than in F7 (+32%) at 500 μM 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 100 μM substrates were used. For comparison of the unique proteolytic pattern of 20 S 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 and 33% higher than in D7 (two-tailed, paired t test; p < 0.005; Fig. 7A). Chymotrypsin- to trypsin-like activity ratios followed a similar proportionality and were on 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 20 S Subunit Compositions across 20 S IEF-FFE Fractions—The results presented in Figs. 6 and 7 demonstrated that 20 S complexes separated by FFE exhibited distinct proteolytic patterns. Substrate specificity as well as turnover of the 20 S proteasomes is dependent on incorporation of constitutive and inducible β1, β2, and β5 subunits because they bear the proteolytic sites. To address whether differences in 20 S assembly contribute to the distinct proteolytic patterns observed in separated 20 S fractions, the concentrations of constitutive and inducible β1, β2, and β5

![Fig. 7. The proteolytic activities of proteasomes with distinct pI values are reproducibly distinguishable at different substrate concentrations. Fractions D7 and F7 from native interval IEF-FFE of cardiac 20 S proteasomes were assayed at substrate concentrations from 25 to 500 μM (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 (bars)). By comparison of the ratios in D7 with 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 confidence for the distance of ratios is shown in A and C.](image-url)
subsequently immunoblotted for the presence of constitutive (\(\beta_1, \beta_2, \beta_5\)) and inducible 20 S subunits (\(\beta_{1i}, \beta_{2i}, \beta_{5i}\)). 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 (bars) are displayed. Quantities of \(\beta_{1i}\) and \(\beta_{2i}\) were significantly different at \(p < 0.05\) (*) and \(p < 0.01\) (**), respectively.

Comparing the amount of proteasome subunits in D7 and F7, all analyzed subunits except \(\beta_{1i}\) and \(\beta_{2i}\) were detected at a similar level (Fig. B8). Interestingly \(\beta_{1i}\) and \(\beta_{2i}\) expressions neither exhibited a similar trend nor were all inducible \(\beta\) subunits changing in unison. Although \(\beta_{1i}\) levels were increased by 47% in D7 (\(p < 0.05\)), \(\beta_{2i}\) was reduced by 58% in F7 (\(p < 0.01\); Fig. B8). This result was unexpected because current 20 S 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 because they occur at much lower amounts than the constitutive subunits (\(~1-5\%)\). 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 20 S complexes suggests the presence of intermediate 20 S proteasomes with mixed assembly of constitutive and inducible subunits. Still the predominant 20 S assembly in cardiac tissue is composed of constitutive subunits. In summary, the results demonstrate that 20 S complexes separated by IEF-FFE have unique compositions, which might contribute to their distinct proteolytic activities.

Different Levels of 20 S 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 pl values between the majority of cardiac and hepatic proteasomes. Because proteasomes purified from both organs contained all known 20 S subunits, no additional associated proteins were detected, and the size of all proteasomes was similar, variations in molecular compositions are unlikely. In contrast, different amounts of additional charge groups, such as phosphorylations, remained undetected in our analysis and potentially could influence the pl significantly. Several 20 S subunits have been reported to be phosphorylated, some of them even at multiple sites (26, 44). Furthermore phosphorylation seems to modulate 20 S proteasome activity (6, 45). Liver 20 S complexes are more acidic than cardiac 20 S proteasomes and possibly phosphorylated to a higher degree. Therefore, 20 S proteasome complexes that are phosphorylated to different extents were produced in vitro to assess their impact on the pl of 20 S proteasomes in native IEF-FFE. The modification of 20 S proteasomes was derived by partial enzymatic dephosphorylation with CIAP. As expected, the pl values shifted after partial dephosphorylation of 20 S proteasomes to the alkaline range (Fig. 9). In total, 20 FFE fractions ranging from pH 5.0 (F53) to 5.58 (F72) contained the characteristic 20 S 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 had 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

![Image of proteasome subpopulations](Image)

**Fig. 8.** Differences in molecular composition of proteasome complexes with distinct pI values. Fractions D7 and F7 were obtained by native interval IEF-FFE of cardiac 20 S proteasomes and subsequently immunoblotted for the presence of constitutive (\(\alpha_3, \beta_1, \beta_2, \beta_5\)) and inducible 20 S subunits (\(\beta_{1i}, \beta_{2i}, \beta_{5i}\)). 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 (bars) are displayed. Quantities of \(\beta_{1i}\) and \(\beta_{2i}\) were significantly different at \(p < 0.05\) (*) and \(p < 0.01\) (**), respectively.

**Fig. 9.** Different phosphorylated forms of hepatic 20 S complexes separated by native IEF-FFE in pH 4–6. Prior to FFE, 20 S proteasomes were 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 are 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. S, unfraccionated sample.
Several reports indicate the co-existence of constitutive proteasomes 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 20 S purifications by a combinatorial approach of 2-DE and MS (for a review, see Ref. 48). Furthermore up to 32 spots on 2-DE gels from 20 S purifications were identified as one of 17 different proteasome subunits, indicating PTMs (26). Several PTMs of proteasome subunits, such as acetylation or phosphorylation, have already been 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 the native protein level. To our knowledge, one single group has demonstrated the presence of 20 S subtypes in single tissues so far (8, 9).

The first indication for additional 20 S subtypes besides the constitutive proteasomes and immunoproteasomes was found in rat skeletal muscle (8). In detail, high resolution ion exchange chromatography of purified 20 S complexes resulted in overlapping but distinct peaks in chromatograms (9). Peaks in chromatograms of 20 S separations obtained from spleen, liver, and kidney were closely overlapping and less pronounced (8). Skeletal muscle-derived 20 S 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 that potentially have different cellular functions or present complexes in different regulatory states. Quantitative alterations of 20 S subtypes have been demonstrated in a disease-related context by studying diabetic rats (49). However, even by using advanced proteomics technologies, the nature of the differences in subtypes remained obscure (29). Targeting of all 20 S 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. (8) state in their report that current 20 S preparations and analyses probably all contain mixtures of 20 S subtypes. For future evaluation of 20 S proteasomes as therapeutic targets, the characterization of subtypes and a description of their function seem to be crucial.

The 20 S 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 immunoproteasome 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 pI seemed not to distinguish between constitutive and immunoprotea-
Mammalian Proteasome Subpopulations

proteasomes, but the presence of intermediate forms is indicated by individually increased or decreased incorporation of β subunits. Therefore, our results support the hypothesis of existing intermediate proteasomes. The pI values of inducible subunits except β5i differ minimally from their constitutive forms, and their influence on the pI values of complexes is presumably minute. In contrast, PTMs are likely to influence the pI values of 20 S complexes because (i) cardiac and hepatic proteasomes both contain all known α and β subunits, but their pI values are significantly different; (ii) hepatic 20 S complexes are distributed over a broader pH range; and (iii) partial dephosphorylation of liver 20 S proteasomes results in complexes with pI values encompassing more than 0.5 pH units. Unknown interacting partners might also influence the pI values of complexes.

Phosphorylation of 20 S subunits seems to regulate multiple mechanisms associated with proteasome structure and activity (48). For instance, phosphorylation modulates the proteolytic activity of cardiac 20 S proteasomes (6), and α7 phosphorylation is associated with 26 S stabilization (8). In the present study, the extent of phosphorylation of 20 S proteasomes has a tremendous effect on its pI, altering it up to 0.5 pH units. Furthermore after partial dephosphorylation of liver 20 S proteasomes, particular dephosphorylated complexes appeared at pI values and distribution similar to those of cardiac 20 S complexes. Therefore, it is conceivable that cardiac and hepatic 20 S proteasomes differ in their extent of phosphorylation. Adding additional support in this matter is that proteasome activity in liver is significantly higher than in heart, and phosphorylation of cardiac 20 S proteasomes promotes its activity (6). It will be of further interest 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 20 S proteasomes (the present study and Refs 8 and 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 most 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 because 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-γ 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 insoluble IEF of multiprotein complexes enabled the separation of distinct native proteasome complexes. Cardiac and hepatic proteasomes displayed unique pI values 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 20 S proteasome populations (1, 50, 51). The possibility to distinguish 20 S proteasomes derived from different tissues as well as within a single organ provides the potential of more accurate therapeutic targeting.

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Mammalian Proteasome Subpopulations


