Published in final edited form as:

Proteomics. 2008 December ; 8(23-24): 5025–5037. doi:10.1002/pmic.200800387.

Two-Dimensional Electrophoresis Based Characterization of Post-translational Modifications of Mammalian 20S Proteasome Complexes

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Abstract

Post-translational modifications (PTMs) serve as key regulatory mechanisms for 20S proteasome functions. Alterations in 20S PTMs have been previously observed with changes in modified protein degradation patterns and altered cellular phenotypes. Despite decades of investigation, our knowledge pertaining to the various PTMs of 20S complexes and their biological significance remain limited. In this investigation, we show that two-dimensional electrophoresis (2-DE) offers an analytical tool with high resolution and reproducibility. Accordingly, it has been applied for the characterization of PTMs including glycosylation, phosphorylation, oxidation, and nitrosylation. The PTMs of murine cardiac 20S proteasomes and their associating proteins were examined. Our 2-DE analyses displayed over 25 spots for the 20S complexes (17 subunits), indicating multiply modified subunits of cardiac proteasomes. The identification of specific PTM sites subsequent to 2-DE were supported by mass spectrometry. These PTMs included phosphorylation and oxidation. Most of the PTMs occurred in low stoichiometry and required enrichment to enhance the detection sensitivity. In conclusion, our studies support 2-DE as a central tool in the analyses of 20S proteasome PTMs. The approaches utilized in this investigation demonstrate their application in mapping the PTMs of the 20S proteasomes in cardiac tissue, which are applicable to other samples and biological conditions.

Keywords

20S Proteasome; Mass spectrometry; Post-translational Modification; Proteolysis; Two-dimensional Electrophoresis

1. Introduction

The 20S proteasome complexes play an essential role for targeted protein degradation, governing the half-life of the majority of cytosolic and nuclear proteins [1]. The substrates for proteasomes are involved in many aspects of cell biology including cell cycle control, apoptosis and ER stress [2]. Therefore, the function of these proteasomes is important for intracellular homeostasis as well as the adaptation to stress.

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The authors have declared no conflict of interest.

The 20S proteasomes are composed of 17 different subunits [3] and is known as the proteolytic core. PTMs of the 20S proteasome modulate its assembly [4], half-life [5] and activities in response to the various biological stimuli of the cell [6]. 20S proteasomes are not uniform and static but instead are generally viewed as versatile and dynamic complexes. Heterogeneous populations were demonstrated to exist in mammalian tissues [7,8], suggesting a diverse role of the proteolytic core and the potential for physiological adaptations. Phosphorylation has been demonstrated to be among the facets that distinguish proteasome subpopulations [8,9], which consequently impact the proteolytic activities [10]. Furthermore, phosphorylation is involved in the switching of associating proteins [4] and reorganizing intracellular traffic [11]. Reversible O-glycosylation has been found to regulate the functionality of the proteasome in response to the cellular metabolic state upstream of phosphorylation events [12]. Under elevated oxidative stress, cell survival has been tied to the functionality of the 20S proteasomes, which is a key mechanism for the removal of oxidatively damaged proteins and for the prevention of the accumulation of toxic aggregates [13]. Poly-ADP ribosylation was found to accelerate the proteasome-dependent turn-over of oxidized histones [14], while modification by 4-hydroxy-2-nonenal (a product of lipid-peroxidation) compromised protein degradation [15]. Many forms of PTMs are shown to modulate proteasome function and thus, the detailed characterization of these modifications will lead to an in-depth understanding of the regulatory switches of proteasomes.

The advancement of proteomic technologies has revolutionized the available tools to address the challenges associated with characterizing PTMs [16]. Two-dimensional electrophoresis (2-DE) resolves proteins based on both isoelectric point and molecular mass [17]. The high resolution of this technology enables the separation and characterization of proteins with PTMs that affect their charge state [18]. 2-DE features high reproducibility, placing it as a central technology for subsequent analyses that identify PTMs [19]. A variety of specific detection technologies have been developed to identify PTMs for 2-DE. In previous studies, the redox state of a cell was monitored through the accumulation of carbonyls and the decrease of free thiols by chemical derivation followed by protein separation on 2-DE [20,21]. Furthermore, antibodies and specific fluorescent stains are available to probe for specific PTMs, such as phosphorylation and glycosylation [22]. Parallel site specific PTM identifications are also supported by the recent advancements in mass spectrometry.

In this manuscript, we characterized PTMs of purified mammalian 20S proteasomes utilizing 2-DE based approaches supported by antibodies, capture reagents and mass spectrometry. 2-DE is presented here as a platform capable of separating various forms of the proteasome subunits, thus enabling the subsequent analysis by PTM specific technologies on individual subunits. Our approach can be easily adapted for comparative analysis in a variety of tissues or conditions as shown previously [10,23]. Large format 2-DE (23cm×30cm) displayed 20S proteasome species from rat liver [23] and mouse colon [24] into more than 49 spots. With smaller format 2-DE gels in conjunction with a capturing agent, a rapid comparative assessment of PTMs is possible with a minimal reduction in resolution. In this study, a list of potential post-translational modification events was examined with capturing agents, including phosphorylation, glycosylation, nitrosylation and oxidation. Using tandem mass spectrometry, sites of post-translational modification were identified on 20S subunits as well as several 20S proteasome associating proteins, including Hsp90 subunit α , 14-3-3 protein γ and eukaryotic translation initiation factor 2- α kinase 4.

2. Materials and Methods

2.1 Materials

Protease inhibitor cocktail (Roche applied science, IN), Sequencing grade modified trypsin (Promega, WI), Phosphatase inhibitor cocktails, BSA (Sigma-aldrich, MO), IPG drystrip gel

pH3-10 non-linear, ECL reagents (GE healthcare, NJ), MonoTip TiO₂ enrichment resin (GL sciences), Pro-Q Emerald glycoprotein stain, Pro-Q Diamond phosphoprotein stain, Sypro Ruby stain (Invitrogen, CA), Anti-nitrotyrosine antibody (Affinity Bioreagents, CA), EZ-Link biotin hydrazide, EZ-Link Iodoacetyl-LC-biotin (Thermo Fisher Scientific, CA), Avidin-HRP (Rockland Immunochemicals, PA), Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC) (Bachem, CA), Oxyblot kit (Millipore, CA).

2.2 20S Proteasome Activity Assay

The peptidase activity of the purified 20S proteasomes from the ICR strain of mouse were assayed with a specific fluorophore-tagged peptide substrate (Suc-LLVY-AMC) targeting the chymotrypsin-like activity of the $\beta 5$ subunit. The assay buffer contains 25mM HEPES, pH 7.5, 0.5mM EDTA, 0.03% SDS. Purified 20S proteasomes were pre-incubated with specific inhibitor (lactacystin) or vehicle for 10 minutes before addition of substrates. Peptidase activities were determined by the release of a free AMC group after cleavage, detected by a Fluoroskan Ascent fluorometer (Thermo Fisher Scientific, MA) (excitation at 390nm and emission at 460nm).

2.3 Two-Dimensional Electrophoresis

Nonlinear IPG strips with a pI range of 3-10 were rehydrated with buffer (8M urea, 0.5% DTT, 0.2% pharmalyte, 1% CHAPS); 340 μ l for 18cm strip and 125 μ l for 7cm strip overnight. 20S proteasomes were precipitated with prechilled 10% TCA in acetone to remove excess salts and subsequently reconstituted with 50 μ l (18cm) or 20 μ l (7cm) of sample buffer (9M urea, 1% DTT, 1% pharmalyte, 2% CHAPS). Isoelectric focusing was conducted on an IPGphor3 manifold (GE healthcare, NJ) following cup-loading protocols (150V, 2 hours; 300V, 2 hours; 600V, 2 hours; 8000V, gradient, 50 minutes; 8000V, with a total Vh of 28,000 for 18cm strip or 150V, 2 hours; 300V, 1 hour; 600V 1 hour; 5000V, gradient, 30 minutes; 5000V, with a total Vh of 8,000 for 7cm strip). The strips were then re-equilibrated sequentially with 1% DTT and 4% iodoacetamide in equilibration buffer (6M urea, 30% glycerol, 2% SDS) for 15 minutes each. Proteins within the equilibrated strips were resolved on 12.5% SDS-PAGE gels. 2-D gels were fixed twice for 30 minutes each with 250ml fixation buffer (50% methanol, 7% acetic acid).

2.4 Antibodies and Capture Reagents

Sypro Ruby was elected to stain proteins resolved by 2-DE, providing excellent visualization of all components of the proteasome complexes. In addition, Pro-Q Emerald 300 dye was elected to probe glycosylation modifications and Pro-Q Diamond dye was elected to detect phosphoproteins. Nitrosylation of 20S proteasome subunits was assessed by immunoblotting with anti-nitrotyrosine antibodies while the EZ-Link Iodoacetyl-LC-Biotin and the Oxyblot were used to examine 20S proteasome subunit oxidation.

Briefly, for glycosylation, the fixed 2-D gels were washed twice (washing solution: 3% acetic acid) for 20 minutes each. Following a 30-minute incubation with 250ml 1% periodic acid in washing solution, the gels were again washed twice with 250ml washing solution for 20 minutes each. Pro-Q Emerald 300 dye was added subsequently for a 90-minute incubation. After destaining with washing solution and rinsing with Milli-Q water, images were acquired with a Gel Doc XR system (UV excitation 280nm, CCD camera 530nm; Biorad, CA). For phosphorylation, Pro-Q Diamond dye was elected to probe phosphoproteins according to the manual. Briefly, fixed 2-D gels were rinsed three times for 10 minutes each with 250ml Milli-Q water. 250ml Pro-Q Diamond dye was then added for a 60-minute incubation. After destaining three times (20% acetonitrile, 50 mM sodium acetate, pH 4.0) for 30 minutes each and rinsing in Milli-Q water, images were acquired with a Typhoon Variable Mode Imager 8600 (excitation 532nm, emission 610nm). For nitrosylation, the 20S proteasome subunits

were assessed by immunoblotting with anti-nitrotyrosine antibodies (Affinity Bioreagents, CA). Proteins resolved by 2-DE were electro-transferred onto a nitrocellulose membrane. After blocking with 3% BSA in TBS overnight, the membrane was incubated with a 1:1000 dilution of anti-nitrotyrosine antibodies for 1 hour. The blot was subsequently probed with HRP conjugated secondary antibody and signaled with ECL reagents. Chemiluminescence was acquired on film and then visualized with a film developer.

For the assessment of protein oxidation, the purified 20S proteasomes were split into two aliquots. One was treated with an ROS releasing agents (10 μ M paraquat, 37°C, 30 minutes) and the other was treated with buffer (no paraquat) as control. Two specific types of oxidative modification: oxidation of the thiol group within the cysteine residue and the accumulation of carbonyl groups, were elected as examples to assess oxidative modification. To assay cysteine oxidation, EZ-Link Iodoacetyl-LC-biotin was added to both parts to the concentration of 100 μ M for 15 minutes at ambient temperature. Proteins were precipitated with TCA/acetone after derivation. Derived proteins were resolved pair-wise on 7cm IPG strips and then displayed on the same 2-D gel as described previously. Biotin tagged subunits were probed with avidin-HRP on a nitrocellulose membrane. To assay carbonyl accumulation by immunoblotting, an Oxyblot kit was used (Millipore, CA). Briefly, 1% (v/v) 2-mercaptoethanol was added to the paired samples. 2,4-dinitrophenylhydrazine (DNPH) was added subsequently. After 15 minutes, the reaction was stopped by neutralizing the mixture. Derived proteins were recovered by TCA/acetone precipitation and resolved pair-wise on 7cm IPG strips. DNP conjugated proteins were probed by immunoblotting with anti-DNP antibodies.

In addition, the carbonyl group accumulation was examined by mass spectrometry, EZ-Link biotin hydrazine was used instead of DNPH. After adding 1% (v/v) 2-mercaptoethanol, the paired 20S proteasome samples were incubated for 2 hours with 5mM EZ-Link biotin hydrazine at ambient temperature. Subsequently, 15mM sodium cyanoborohydride was added for 15 minutes to convert the derivatives into mass spectrometer compatible forms. Excess reagents were removed by TCA/acetone precipitation.

2.5 Protein/Peptide Fractionation for Phosphorylation Analysis

One-dimensional SDS-PAGE or IPG-strip IEF was conducted for sample separations before mass spectrometry. For 1D SDS-PAGE, a preparative-scale 15% T 3.3% C poly-acrylamide gel was used. 100 μ g of 20S proteasomes were denatured and then resolved. The gel was fixed and stained with CBB, with the gel bands excised for individual analyses. The in-gel isoelectric focusing was conducted on 18cm 3-10 non-linear IPG strips as described previously. Proteins were digested in-gel before mass spectrometry characterization. Complete endoproteolytic digestions were carried out with trypsin (1:50 as the ratio of enzyme versus proteasomes in 50mM ammonium bicarbonate pH 8.0) overnight at 37°C. Partial endoproteolytic digestion was carried out with trypsin (1:100 as the ratio of enzyme versus proteasomes in 50mM ammonium bicarbonate pH 8.0) at 37°C for 3 hours. Peptides were then extracted from the gel.

Phosphopeptides were enriched with TiO₂ resin. TiO₂ MonoTips were sequentially equilibrated with 3 solutions: 50mM AmBC containing 25% ACN, 50% ACN solution containing 5% TFA (v/v) and lastly 50% ACN solution containing 0.1% TFA (v/v). Dried peptides were dissolved with 50% ACN containing 0.1% TFA (v/v), loaded onto the TiO₂ resin with 80 repetitions, and subsequently rinsed twice with 10 cycles of 100 μ l of 50% ACN solution containing 0.1% TFA. Finally, phosphopeptides were recovered with 3 sequential elution solutions, 100 μ l of 50% ACN solution containing 5% TFA (v/v), 50mM ammonium bicarbonate containing 20% ACN and lastly 1% ammonia. All elution fractions were dried and then subjected to mass spectrometry analyses.

2.6 Mass Spectrometry Based Characterization of PTMs

Further mass spectrometry analyses supported the site specific characterization of both the phosphorylation peptides and the oxidation modifications. Briefly, the reconstituted peptide mixtures were analyzed by LTQ linear ion-trap in either the CID mode or the ETD mode (Thermo Fisher Scientific, CA). The flow rate for nano-RPLC was set to 5 μ l/min for peptide loading and 220nl/min for separation (buffer A: 0.1% formic acid, 2% ACN; buffer B: 0.1% formic acid, 80% ACN). A linear gradient of 100 minutes was set between 2% buffer B to 50% buffer B for peptide separation. Data-dependent acquisition was set as one full MS scan was followed by five MS² scans. Acquired spectra were searched against the IPI mouse database (Version 3.34 with 51424 entries) using the SEQUEST algorithm (Bioworks v.3.3.1 was used for the .dta file generation).

In CID mode, the search parameters for the various PTM identifications were set as the following: partial enzymatic digestion (trypsin), permitting two miscleavages, peptide tolerance as 2.0, and fragment tolerance as 1.0. In parallel, fixed modification of cysteine was set at +57 Da, differential modifications of methionine oxidation was set at +16 Da; phosphorylation of serine, threonine and tyrosine was set at +80 Da; water loss of serine and threonine was set at -18 Da; and protein N-terminal acetylation was set at +42 Da. The differential modification parameters for carbonyl conjugation with biotin hydrazide were set as the following: methionine oxidation was set at +16 Da; biotinylation of threonine was set at +242 Da; biotinylation of arginine was set at +201 Da; biotinylation of lysine was set at +243 Da; and biotinylation of proline was set at +260 Da. The differential modification parameters for thiol conjugation were set as the following: methionine oxidation was set at +16 Da; biotinylation of cysteine was set at +383 Da and carbamidomethylation of cysteine was set at +57 Da. Identified peptides were filtered according to the following criteria: Xcorr > 2.5 (+2), 3.5 (+3) [25]. Manual spectra inspection was conducted to eliminate false positives. In ETD mode, the search parameters for phosphorylation identification were set as the following: partial enzymatic digestion (trypsin), permitting three miscleavages, peptide tolerance as 2.0, and fragment tolerance as 1.0. In parallel, fixed modifications of cysteine was set at +57 Da; dynamic modifications of methionine oxidation was set at +16 Da; the phosphorylation of serine, threonine and tyrosine was set at +80 Da; the water loss of serine and threonine was set at -18 Da; and protein N-terminal acetylation was set at +42 Da. Identified peptides were filtered according to the following criteria: Xcorr > 2.0 (+2), 2.5 (+3), 3.0 (>+3) [26]. Manual spectra inspection was conducted to eliminate false positives.

3. Results and Discussion

3.1 2-DE Based Characterization of 20S Proteasome PTMs

In this investigation, a total of four types of PTMs were identified on multiple proteasome subunits and their associating partners (summarized in Table 1). These include phosphorylation, glycosylation, oxidation, and nitrosylation. Since biochemical characterization of PTMs is primarily based on specific staining or antibody affinity to the PTM moieties, it is crucial to separate the sample to the level of individual proteins to unambiguously assign a specific signal to a single protein. To achieve this goal, we applied different separation technologies to resolve proteins down to single species. In this regard, 2-DE enabled simple visualization by staining and was compatible with numerous proteomic technologies, such as immunoblotting or mass spectrometry. The flowchart of our approach is outlined in Figure 1. This approach resolved denatured proteins based on their isoelectric points and molecular masses [18]. The murine cardiac 20S proteasomes were purified and their integrity (Fig. 2A) and function (Fig. 2B) confirmed. These protein complexes were separated by 2-DE, as the resolution supported the separation of 20S proteasome subunits into distinct forms (Fig. 2C). Furthermore, our results showed that the reproducibility of 2-DE was

consistent with previous reports [19]. Large format 2-DE (23cm×30cm) has been employed to display 20S proteasome species in an organ-specific fashion (liver, small intestine, colon, spleen, and thymus), with up to 50 spots stained [23]. The employment of capturing agents and smaller format gels (18cm×14cm or 7cm×14cm) enabled the rapid probing of PTMs. To enable reproducibility and comparability in our investigation, special attention was given for proper sample preparation (see details below). 2-DE separated and displayed multiple subunits of 20S proteasomes; among them, $\alpha 1$, $\alpha 3$, $\alpha 4$, $\alpha 6$, $\alpha 7$, $\beta 2$, $\beta 5$ and $\beta 6$ were represented by more than a single spot, indicating possible PTMs of those subunits (Fig. 2C).

3.1.1 Sample Preparation and 2-DE Separation—The murine cardiac 20S proteasomes represent a sub-proteome of the cardiac cell. 20S proteasomes were purified according to a previously published protocol [10]. The purified 20S proteasomes were stable complexes shown by electron microscopy in Figure 2A, with fractions of the purified complexes demonstrating proteolytic activities (Figure 2B). The preparation assured a clear separation of the subunits and associating partners on the 2-DE. Variances exist among analyses originating from different species, tissues and between various age groups. A collective presentation of these variance aids in the identification of potentially important regulatory events. For a detailed description of 2-DE sample preparation, publications by Gorg. et al. are recommended [18].

The cardiac 20S proteasome complexes are composed of 17 distinct subunits, all which are hydrophilic and smaller than 30 kDa. The standard urea sample buffer as published by Gorg et al. [18] was sufficient for solubilizing the proteasome subunits for 2-DE. The addition of thiourea or particular detergents was not necessary for high quality separation. When 20S proteasome samples were subjected to 2-DE directly after ion exchange chromatography, TCA/acetone precipitation was effective for the removal of salts which interfere with focusing at concentrations higher than 30mM.

In our 2-DE map (Fig. 2C), subunits of the murine 20S proteasome ranged from pIs of 4.7 ($\alpha 5$) to 8.9 ($\alpha 4$), consistent with their in-silico analyses (www.expasy.org/cgi-bin/pi_tool). Subunits bearing the proteolytic sites were all subject to N-terminal truncation during proteasome assembly [27], which largely affected their pIs. PTMs such as phosphorylation, also affect their pIs. Some of the proteasome subunits were quite alkaline, to which the application of immobilized pH gradient (IPG) strips aided their separation [18]. Non-linear IPG strips covering a pH range from 3-10 were used for the separation of murine 20S proteasomes, their shallower pH gradient in the range more complimented the focusing of proteasome subunits. The existence of multiple forms of a given subunit was better demonstrated on an 18 cm strip as shown in figure 2 than on a 7 cm strip (Fig. 3). However, running short strips and combining them with SDS mini-gels enabled a quick comparison of PTM profiles of multiple biological samples (Fig. 3), thus, minimizing gel-to-gel variations. Highest quality and reproducibility in comparative 2-DE analyses was achieved by pre-electrophoretic labeling of different samples with up to three fluorescent tags utilizing the 2-D DIGE technology [18]. The labels affect pIs and MW similarly, enabling mixing and co-separation on 2-D gels. Subsequently, the protein patterns were visualized via excitation of the three different fluorescent labels. Inclusion of an internal standard facilitates the highest reproducibility for the quantitation of biological expression patterns.

We optimized our staining approach using the following criteria: equivalent staining of unmodified and PTM containing proteins, highly sensitive detection of low abundant proteins, a wide dynamic detection range to visualize both low and high abundant forms and compatibility with subsequent mass spectrometric identification. SYPRO Ruby was selected and it stained all classes of proteasome proteins, including phosphoproteins and glycoproteins without a detectable bias. This stain was sensitive at levels significantly superior to CBB

staining and was comparable to silver staining. Overnight incubation of this dye with proteins displayed via 2-DE provided the highest sensitivity. The linear dynamic range was over three orders of magnitude, thus supporting the visualization of lowly abundant forms without over-staining highly abundant proteins. The SYPRO Ruby fluorescent dye stained proteins via the association of a ruthenium-containing organic complex, forming a non-covalent interaction that was desirable for subsequent mass spectrometry analyses [28]. This contrasted to silver stain procedures which introduce a formaldehyde cross-linking reagent. The sensitivity, dynamic range and lack of bias of SYPRO Ruby combined with its compatibility with mass spectrometry made this stain ideal for the visualizing 20S proteasome PTMs. SYPRO Ruby staining displayed all murine 20S proteasome proteins and was used to normalize quantitative PTM analysis (Fig. 3D).

3.1.3 Identification of PTMs in the 20S Proteasome Complexes

a. Glycosylations: The detection of glycosylated 20S subunit was achieved by using a reagent that specifically targeted carbohydrate moieties. There are at least two principle biochemical approaches developed for glycoproteomics. One approach is a reagent that takes advantage of lectin's binding to specific carbohydrates, the specificity of this dye allows for subclasses of glycoproteins to be differentiated. The other approach utilizes periodate/Schiff's base chemistry to generate a general stain towards glycoproteins. Periodic acid converts the glycols within glycoproteins to aldehydes, which are then potentiated for tagging. We applied Pro-Q Emerald 300 for the identification of 20S proteasome glycosylation, which operated by conjugating to the aldehyde moiety that is capable of emitting a strong green fluorescence once bound [29]. This approach achieved speedy and sensitive in-gel staining (Figure 3A). Repeated fixation and washing steps were implemented to eliminate SDS from the gel. Subunits $\alpha 1$, $\alpha 2$, $\alpha 3$, $\beta 4$, $\beta 5$ and $\beta 6$ of the murine cardiac 20S proteasome responded to Pro-Q Emerald 300 staining. This represented the first report of glycosylation of 20S subunits in mammalian cells (Fig. 3A).

b. Phosphoprotein Staining: We used the Pro-Q Diamond stain for phosphoprotein staining. This approach is capable of globally probing the steady-state phosphorylation of 20S proteasomes (Figure 3B). There has been a report that phospholipids introduce non-specific staining by Pro-Q Diamond; however, in our studies, there was no evidence to support that phospholipids exists in the 20S proteasomes purified via multi-dimensional chromatography. The removal of electrolytes before staining provides additional insurance against any interfering with the staining procedure [30]. Our results showed that a reproducible image acquisition required particular attention to the incubation time with the dye as extended staining regimens can compromise specificity. Phosphoprotein specific staining with Pro-Q Diamond heavily stained 20S proteasome subunit $\alpha 7$, which was potentially phosphorylated at multiple sites or phosphorylated at high stoichiometric ratio at one site, while subunits $\alpha 1$, $\alpha 2$, and $\beta 6$ were more weakly stained, indicating less phosphorylation (Fig. 3B).

c. Nitrosylated Proteins Immunoblotting: Nitric oxide is an important messenger for intracellular signaling, especially in the heart. Physiological as well as pathological regulatory events correlate with the release of NO. Tyrosine residues are susceptible to NO induced modification that generate nitrotyrosine, a suitable epitope for antibody recognition [31]. In our studies, a significant level of nitrotyrosine were stained (Figure 3C). To minimize background staining, the 2-DE displaying proteasomes underwent overnight blocking of the transblot with 3% BSA and extensive washing after incubation. Probing 2-DE displayed 20S subunits with anti-nitrotyrosine antibodies, subunits $\alpha 1$, $\alpha 2$, $\alpha 7$, $\beta 1$, $\beta 3$, $\beta 5$ and $\beta 7$ exhibited positive signals (Fig. 3C).

d. Oxidized Protein Survey: Under elevated oxidative stress, 20S proteasomes not only turn-over oxidized protein substrates but are also themselves the subject of oxidative modification

[15]. The sulfhydryl group of the cysteine residue is among the most easily oxidized functional groups of amino acid side chains. The sheer number of potential sites on the average protein makes the accumulation of carbonyl groups the most sensitive marker of oxidative damage [32]. We elect the depletion of thiol groups and the accumulation of carbonyl groups as the two model markers for surveying the redox status of 20S proteasomes.

A main challenge associated with the study of 20S proteasome oxidation is the differentiation of primary oxidation events associated with biological events and the secondary oxidation introduced nonspecifically during sample preparation. A possible remedy is to use a chemical reagent to derive the primary oxidation product into a form that is distinct from the secondary oxidation products. To accomplish this goal, reagents targeting oxidation events were added at the beginning of sample preparation before secondary oxidation can occur or subsequent to the addition of anti-oxidant compounds [33].

e. Immunoblotting Analysis of Free Thiol Groups: The oxidation of cysteine residues at catalytic centers will frequently inactivate an enzyme. Cysteine residues can be oxidized into multiple forms including cystine, sulfenic, sulfinic and sulfonic acids. It is technically challenging to monitor simultaneously and quantitatively all of these oxidation products within one sample. Examination of the level of free thiol groups represents an alternative strategy to survey the extent of thiol group oxidation. Iodoacetamide is a sulfhydryl reactive compound widely used in proteomics, including 2-DE and in-gel digestion for mass spectrometry. Biotin conjugated iodoacetamide (BIAM) selectively introduces a biotin tag to free thiol groups and forms an adduct that is easily analyzed via immunoblotting or mass spectrometry. Upon paraquat treatment for example, subunits $\alpha 2$, $\beta 1$, $\beta 3$ and $\beta 5i$ were oxidized at higher levels (Fig. 4A).

f. Immunoblotting Analysis of Carbonyl Accumulation: Carbonyl groups form from oxidized products of arginine, lysine, proline and threonine residues, representing the most abundant oxidation marker [32]. The generation of carbonyl groups represents a non-reversible form of protein damage. Accumulated carbonyl groups are not affected by supplement of the anti-oxidant 2-mercaptoethanol, which is used to prevent secondary oxidation. 2,4-dinitrophenylhydrazine selectively derives carbonyl groups into immuno-reactive 2,4-dinitrophenylhydrazone for antibody detection (Figure 4B). In the standard derivation reaction, SDS is added to a final concentration of 6% to ensure complete protein denaturation. High concentrations of SDS during the derivation appear to affect the quantitative recovery of 20S proteasomes during subsequent TCA/acetone precipitation. Therefore, SDS was omitted during the derivation reaction of 20S proteasomes prior to 2-DE analysis. The derivatives were stable during TCA/acetone precipitation and any subsequent 2-DE separation. An increase in carbonylation of 20S proteasome subunits was detected for the $\alpha 2$, $\alpha 4$, $\alpha 6$ and $\beta 3$ subunits after ROS treatment.

3.2 Characterization of 20S Proteasome PTMs by Mass Spectrometry

Subsequent to the 2-DE analyses, mass spectrometry analyses were conducted to verify some of the PTMs. One limitation of mass spectrometry lies with the limited dynamic detection range that is unable to match the dynamic range of proteins found within biological systems [34]. In Figure 3, the $\alpha 7$ subunit was more heavily stained by the phosphoprotein dye when compared to other subunits, illustrating the wide dynamic range of phosphorylation on 20S proteasome subunits. In our studies, this challenge was alleviated by increasing the amount of sample applied and through protein fractionation. This succeeded in elevating potentially obfuscated targets within each fraction to within the detection range of the mass spectrometer.

Mass spectrometers examine PTMs of the 20S proteasomes by monitoring the signature mass shifts introduced to peptides [35]. A signature mass shift from a PTM needs to be specific and stable for analysis in order for it to be examined directly by a mass spectrometer. In respect to 20S phosphorylation, an +80 Da shift is introduced to the modified serine, threonine or tyrosine residues. For proper survey of oxidative modifications of 20S proteasomes, chemical derivation reactions are necessary to obtain an accurate and stable signature.

3.2.1 Oxidized Protein Characterization with Mass Spectrometry—To characterize the oxidation status of 20S proteasomes, it is important to differentiate the primary oxidation of biological significance to the secondary oxidation introduced during sample preparation. One approach towards minimizing this effect utilizes a chemical reagent that targets free thiol groups and is added early in the first step of sample preparation before any secondary oxidation events can occur. A mass shift of 382.53 Da is introduced to non-oxidized cysteine residues and can easily be detected via a mass spectrometer. Cysteine¹⁸⁵ of proteasome subunit β 1 (KDECLQFTANALALAMER) was identified after iodoacetyl-LC-biotin labeling and subsequent analysis by an LTQ LC-MS/MS mass spectrometer (Fig. 5A).

Reagents such as DTT or the more stable 2-mercaptoethanol were added before the supplemental derivation reagent to label the accumulated carbonyl groups. For example, when 20S proteasome oxidation was stimulated by the ROS releasing reagent 1% (v/v) 2-mercaptoethanol, secondary oxidation was sufficiently blocked, with no effect on the detection of accumulated carbonyl groups. DNPH derivation was used for immunoblot analysis; however, as the derivation product is not compatible with mass spectrometry analyses. The alkaline solution used during in-gel digestion promoted the hydrolysis of DNPH derivatives. Sodium cyanoborohydride, which is commonly used to stabilize hydrazones, cleaves the DNPH derivatives as well. Substitution of DNPH with EZ-Link biotin hydrazide in the derivation step generated a conjugate that can be subsequently stabilized with sodium cyanoborohydride. The resulting end product was mass spectrometer compatible.

Carbonyl groups accumulate on multiple residues (arginine, lysine, proline and threonine) under elevated levels of oxidative stress through a variety of chemical pathways [32]. As a result, differential mass shifts are introduced to these respective residues. During biotinylation, a biotin tag is conjugated to any carbonyl groups acquired via primary oxidation. Sodium cyanoborohydride reduction introduces two hydrogen atoms to the biotinylated product yielding a signature mass shift (a combination of carbonyl formation, biotinylation and reduction) of +242.33 Da for threonine, +201.07 Da for arginine, +243.31 Da for lysine and +260.34 Da for proline. Threonine 94 of the proteasome subunit β 7 (DS~~T~~MLGASGDYADFQYLK) was identified by mass spectrometry after derivatization using biotin hydrazide (Fig. 5B).

3.2.2 Characterization of Phosphorylation on the 20S Proteasome Associating Proteins—A challenge associated with studying phosphorylation is the low abundance of its occurrence [25], the identification of which benefits from an enrichment procedure targeting phosphoproteins. Compared to 20S proteasome subunits, proteasome associating proteins exist at lower stoichiometry within the complexes. The higher sample capacities of SDS-PAGE and in-gel IEF enabled the characterization of phosphorylation on 20S proteasome associating proteins. Co-migration of different proteins is inherent to high throughput sample fractionation approaches with lower resolution which often leads to competitive binding with the resin and the loss of less abundant species. Implementation of various fractionation approaches minimized the loss of signal (Figure S1). Complementary data can be collected by the combination of sample fractionations. The differential display of proteasome complexes by these two approaches each contributes to a distinctive set of identifications.

Sensitive capture of phosphopeptides required maximizing enrichment efficiency. The enrichment protocol was optimized using a known 20S phosphopeptide as a positive control, the C-terminal peptide of the $\alpha 7$ subunit (ESLKEEDES_pDDDNM) [36]. A combination of acids has been evaluated for different biological samples. Improvements in specificity were often observed at the expense of the recovery rate. Typically 2,5-dihydroxybenzoic acid (DHB) is supplemented in the loading and washing buffers for TiO₂-resin based phosphopeptide enrichment to increase specificity but was not included in this protocol as this known phosphopeptide was observed in the flow through and washing fractions when DHB was supplemented. Additional care was taken since the phosphate moieties are susceptible to β -elimination in the mass spectrometer or alkaline solution. Usually an alkaline buffer is used to elute phosphopeptides in the final step of the enrichment. In this case, however, we established a three-step elution procedure that maximizes phosphopeptide recovery. The first elution with an acidic solution decreases the resin-binding affinity of phosphopeptides by inhibiting the deprotonation of the phosphate group. Ammonium bicarbonate in the second elution buffer competes with the phosphopeptide ions bound to the resin. A final elution with alkaline buffer enhances the recovery of the remainder phosphopeptides.

ETD and CID each offered unique strengths in phosphopeptide identification. In CID mode, peptide precursors are brought to resonant vibration, during which, collisions with helium gas build up thermal energy. Peptides start to fragment when the energy reaches a threshold. Resonant vibration ensures the complete fragmentation of peptide precursors thus generating peaks for sequencing. Peptides possessing double or multiple charges are all be efficiently fragmented. A limitation of CID in phosphorylation characterization is the vulnerability of the labile phosphate moiety, which are often removed during the buildup of vibrational energy. ETD provides an alternative fragmentation scenario in which fragments are generated without breaking the phosphate-peptide bond. ETD fragmentation favors precursors with more than two positive charges. For doubly charge ions, fragmentation is less efficient. For a detailed description of ETD, publications from Coon are recommended [26]. It has previously been established that CID and ETD have distinct biases in peptide sequencing, making it advantageous to combine the approaches to detect phosphorylation of 20S proteasome associating proteins.

The implement of the above optimized phosphorylation identification platform provided fertile phosphopeptide identifications for several 20S proteasome associating proteins. 20S proteasome associating proteins existed in substoichiometric amounts within the purified 20S proteasome complexes, complicating the detection of phosphopeptides. HSP90 is a known interacting partner of 20S proteasomes that affects proteolytic activities as well as susceptibility to oxidative stress [37,38]. HSP90's proteasome specific function was also found to be affected by phosphorylation [39]. A phosphorylated form of the HSP90 D subunit at serine²⁶³ has now been identified (Figure 6A). 14-3-3 proteins have also been previously reported to associate with 20S proteasomes [40]. It is capable of serving as an adaptor protein, recognizing specific phospho-serine or phospho-threonine motifs. Threonine³¹ was identified in the phosphorylated form of 14-3-3 γ associated with 20S proteasomes (Figure 6B), while the functional impact of this modification has yet to be defined. Eukaryotic translation initiation factor 2- α kinase 4 (Eif2ak4) is another 20S associating phosphoprotein that has been identified by ETD-based mass spectrometry (Figure 6C). Eif2ak4 regulates the α subunit of Eif2 and may mediate translational control. This observation suggests the existence of a molecular mechanism to balance protein synthesis and degradation in eukaryotes [41], perhaps similar to the ubiquitin precursor that is expressed as a ubiquitin and a ribosomal subunit fusion protein.

4. Concluding Remarks

A 2-DE centric approach was implemented for the characterization of various PTMs among the subunits and associating proteins of the 20S proteasomes. Multiplex detection systems enabled the visualization of these PTMs on a single 2-D gel or 2-D blot showing more than 1000 proteins and by streamlining PTM analyses. Mass spectrometry analyses were applied to support the 2-DE characterization. With the application of ETD and CID, a significant number novel PTM sites were identified. In our studies, we see the advantage of 2-DE in the separation of different charge or mass forms of a single protein and the gaining of quantitative data of these forms under various conditions without the necessity of identifying PTMs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported, in part, by NIH grants HL R01-63901, HL R01-65431 and HL P01-80111 to P. Ping; AHA #0715004Y to GW. Young; AHA #0625062Y to O. Drews, and by an endowment from Theodore C. Laubisch at UCLA to P. Ping.

Abbreviations

20S proteasomes, proteolytic core particles of proteasomes; ADP, Adenosine 5'-diphosphate; ACN, acetonitrile; DHB, 2,5-dihydroxybenzoic acid; DNPH, 2,4-dinitrophenylhydrazine; AMC, 7-Amino-4-methylcoumarin; CID, collision-induced dissociation; ETD, electron-transfer dissociation.

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Figure 1. Overview of Available Technologies Presented in this Manuscript to Analyze PTMs of 20S Proteasomes after 2-DE

Over numerous analyses, 2-DE based separation of 20S proteasome subunits was implemented to visualize as well as to characterize various types of PTMs. Post-translationally modified subunits were visualized on 2-DE gels by specific chemical-derivation reagents, PTM sensitive dyes, or antibodies. The approach allowed for the parallel identifications of the modifications and the modified proteins to be identified by advanced mass spectrometry, such as LTQ-ETD tandem mass spectrometry.

Figure 2. Structurally Intact and Functional 20S Proteasomes Are Multiply Modified as Shown by 2-DE

A) The structural integrity of the purified cardiac 20S proteasomes was shown by electron microscopy after negative staining. Images at 59,000x magnification displayed 20S proteasome complexes with their prominent barrel structure of four-stacked rings. B) The functionality was demonstrated by its sensitivity to the specific proteasome inhibitor lactacystin. * defines peptidase activity significantly lower than that of vehicle-treated control group; $P < 0.05$. C) 20S proteasomes were separated by 2-DE on 18 cm, 3-10 NL IPG strips (T 12.5%, C 3.3%) and SYPRO Ruby stained. Multiple subunits ($\alpha 1$, $\alpha 3$, $\alpha 4$, $\alpha 6$, $\alpha 7$, $\beta 2$, $\beta 5$ and $\beta 6$) were represented by more than a single spot, indicating possible PTMs of those subunits. All results were obtained from purified murine 20S proteasome complexes.

Figure 3. Quantitative Visualization of Glycosylation, Phosphorylation and Nitrosylation of 20S Proteasome Subunits after 2-DE

20S complexes were run in parallel on 7cm, 3-10NL IPG strips (T 12.5%, C 3.3%) and stained or blotted for specific PTMs. Images A-C show overlays with quantitative staining of total protein by SYPRO Ruby shown in D. A) The glycoprotein specific dye, Pro-Q Emerald 300, indicates that murine 20S proteasomes are glycosylated on subunits $\alpha 1$, $\alpha 2$, $\alpha 3$, $\beta 4$, $\beta 5$ and $\beta 6$ (red to green in overlay). B) Phosphoprotein specific staining with Pro-Q Diamond indicates that the murine 20S proteasome subunit $\alpha 7$ was potentially phosphorylated at multiple sites or at high stoichiometry while subunits $\alpha 1$, $\alpha 2$, and $\beta 6$ were more weakly stained, indicating less phosphorylation (red to green in overlay). C) Murine 20S subunits separated by 2-DE bound anti-nitrotyrosine antibodies after immunoblotting, indicating that subunits $\alpha 1$, $\alpha 2$, $\alpha 7$, $\beta 1$, $\beta 3$, $\beta 5$ and $\beta 7$ were nitrosylated (red to green in overlay). D) SYPRO Ruby staining display of total protein after 2-DE of murine 20S proteasomes, which was used to normalize quantitative PTM detection (shown in green in A-C).

Figure 4. Detection of 20S Proteasome Subunits Modified by Reactive Oxygen Species after 2-DE Images were taken from a study on a differential analysis of ROS sensitive 20S proteasome subunits, which were characterized using biotin-conjugated chemical reagents to visualize the degree of oxidation. A) Free thiol groups of murine 20S proteasome subunits were conjugated with iodoacetyl-LC-Biotin and probed with avidin-HRP. In this indirect assay, diminishing signal indicates an increasing level of oxidation. Parallel detection of free thiol groups in vehicle and ROS treated murine proteasome complexes demonstrated that the α 2, β 1, β 3 and β 5i subunits were oxidized at higher levels after ROS treatment. B) In parallel experiments, carbonylation was visualized by derivatization with 2,4-dinitrophenylhydrazine (DNPH) and probed with an anti-DNP antibody. In this direct assay, the increasing signal indicates higher levels of oxidation. Increased carbonyl modification of 20S proteasome subunits was detected for α 2, α 4, α 6 and β 3 after ROS treatment.

Figure 5. Identification of Oxidation Sites on Murine 20S Proteasome Subunits by Mass Spectrometry

Chemical derivatization (biotinylation) was employed as a mass tag that was specific to signal protein oxidation. This approach stabilized the oxidative state of the proteins and facilitated oxidation site identification unambiguously by mass spectrometry. In the left panel, the free thiol group on Cysteine¹⁸⁵ of the β1 subunit was identified after iodoacetyl-LC-biotin labeling and subsequent analysis by an LTQ LC-MS/MS mass spectrometer (b marks the biotinylated residue; * marks a H₂O loss peak). Cysteine¹⁸⁵ was detected with a mass shift of 382.53 Da, indicating biotinylation. The complete sequence of the peptide was similarly derived by fragmentation and identified the peptide as KDECLQFTANALALAMER. In the right panel, a carbonyl group located on Threonine⁹⁴ of the β7 subunit was identified after derivatization with biotin hydrazide. Oxidation on the Threonine residue was detected through a mass shift of 242.33 Da in the derived sequence of DSTMLGASGDYADFQYLK (b marks the biotinylated residue; * marks the H₂O loss peak).

Figure 6. Identification of the 20S Proteasome Interaction Phosphoproteins

A) HSP90 α subunit derived phosphopeptide was detected from IEF resolved 20S complexes via CID on an LTQ mass spectrometer. B) 14-3-3 protein γ subunit derived phosphopeptide was obtained from SDS-PAGE resolved 20S complexes via CID on a LTQ mass spectrometer. C) Eifak4 derived phosphopeptide was sequenced from SDS-PAGE resolved 20S complexes via ETD on an LTQ mass spectrometer. # denotes neutral loss of phosphate group. *denotes water loss peak.

Table 1

Detection of PTMs within Murine Cardiac 20S Proteasome Complexes.

Detection	Glycosylation	Phosphorylation	Nitrosylation	Oxidation
Antibodies or capture reagents	2-DE + Pro-Q Emerald 300 ($\alpha 1$, $\alpha 2$, $\alpha 3$, $\beta 4$, $\beta 5$ and $\beta 6$ subunits)	2-DE + Pro-Q Diamond ($\alpha 1$, $\alpha 2$, $\alpha 7$ and $\beta 6$ subunits)	2-DE + anti-nitrotyrosine ($\alpha 1$, $\alpha 2$, $\alpha 7$, $\beta 1$, $\beta 3$, $\beta 5$ and $\beta 7$ subunits)	2-DE + iodoacetyl-LC-biotin ($\alpha 2$, $\beta 1$, $\beta 3$ and $\beta 5i$ subunits) 2-DE + DNPH ($\alpha 2$, $\alpha 4$, $\alpha 6$ and $\beta 3$ subunits)
Mass spectrometry		IEF + LTQ-CID (<i>HSP90α</i>)		2-DE + iodoacetyl-LC-biotin + LTQ-CID ($\beta 1$ subunit)
		SDS-PAGE + LTQ-CID (<i>14-3-3 protein γ</i>)		2-DE + biotin hydrazide + LTQ-CID ($\beta 7$ subunit)
		SDS-PAGE + LTQ-ETD (<i>Eifak4</i>)		