Mapping and Structural Dissection of Human 20 S Proteasome Using Proteomic Approaches*

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The proteasome, a proteolytic complex present in all eukaryotic cells, is part of the ATP-dependent ubiquitin/proteasome pathway. It plays a critical role in the regulation of many physiological processes. The 20 S proteasome, the catalytic core of the 26 S proteasome, is made of four stacked rings of seven subunits each (α7β7α7). Here we studied the human 20 S proteasome using proteomics. This led to the establishment of a fine subunit reference map and to the identification of post-translational modifications. We found that the human 20 S proteasome, purified from erythrocytes, exhibited a high degree of structural heterogeneity, characterized by the presence of multiple isoforms for most of the α and β subunits, including the catalytic ones, resulting in a total of at least 32 visible spots after Coomassie Blue staining. The different isoforms of a given subunit displayed shifted pI values, suggesting that they likely resulted from post-translational modifications. We then took advantage of the efficiency of complementary mass spectrometric approaches to investigate further these protein modifications at the structural level. In particular, we focused our efforts on the 7 subunit and characterized its N-acetylation and its phosphorylation site localized on Ser250.

Molecular & Cellular Proteomics 1:567–578, 2002.

The 20 S proteasome is the catalytic core of the 26 S proteasome complex that represents the major component of the ATP/ubiquitin-dependent protein degradation pathway. This pathway is responsible for the degradation of intracellular proteins tagged with mult ubiquitin chains and thus regulates many processes in the cell such as progress through the cell cycle, gene transcription, and metabolic pathway (for a review see Rock and Goldberg (1)). Misfolded, damaged, mutant, or viral proteins are also digested into shorter peptides that are subsequently degraded by other cytosolic proteases to provide a continuous source of amino acids. A fraction of the peptides generated by the proteasomal proteolysis escapes this further degradation and is presented, associated to major histocompatibility class I molecules, at the cell surface where they can activate the immune system (2).

20 S proteasome structures and functions have been extensively and intensively studied this last decade (3). Present both in the cytoplasm and the nucleus of eukaryotic cells, the 20 S proteasome is a 700-kDa barrel-shaped particle arranged in four stacked rings of seven subunits each, α7β7α7, with molecular masses ranging from 22 to 31 kDa. The subunits are classified as α- and β-type subunits based on their homology to the subunits of the simpler proteasome from Thermoplasma acidophilum (4). The active proteolytic sites are located in the β subunits that constitute the two inner rings of the particle (5). The two outer α subunit rings participate in proteasome assembly (6), control substrate entry (7), and associate with regulatory particles such as PA28 and PA700 (8, 9). Upon induction by interferon-γ, the catalytic subunits β1, β2, and β5 can be replaced by three other subunits β1i, β2i, and β5i, respectively, to form the “immuno-proteasome” that exhibits a modified activity profile as compared with that of the constitutively expressed standard proteasome (3, 10, 11). In human tissues, the 20 S proteasome has also been shown to be present as a mixture of proteasome subtypes possessing different catalytic activities (12). Multiple isoforms of proteasome subunits have been described (13–15), which further account for the structural complexity in 20 S proteasome subunit composition.

Aside from the physiological difference between standard and immunoproteasome activities, the proteasome catalytic activity may be affected by various environmental factors such as oxidative stress, pathological states such as cancers or neurological disorders, aging, or pharmacological agents (16). The impact of structural modifications and more particularly post-translational modifications that affect either the protein to be degraded or the proteasome subunits may lead to altered or even inhibited proteolytic functions (17). Glycolysis (18–20), N-acetylation (21–23), 4-hydroxy-2-nonenal alkylation (24), and phosphorylation (25–30) that affect 20 S proteasome from different species or organisms (human, rat, yeast, and plants) have been evidenced.

Given its implication as a vital element of the cellular metabolism, in the control of cell cycle or tumor growth, the human proteasome is now considered as a new pharmaco-
logical target (31). Indeed, intense efforts of research are currently made for (i) the search of inhibitors as research tools or drug candidates for cancer therapy (32–34) and (ii) the functional exploration of the human 20 S proteasome from cancer (versus normal) cells (35). Data obtained from these studies must be correlated to and validated by comparative analyses of the proteasome structure and/or composition between normal and pathological (cancer) cells. However, such structural comparative studies have not yet been established. A mandatory step to allow further progress in this field is the knowledge of the precise structural identity of the human 20 S proteasome subunits.

Recent advances in mass spectrometry for the analysis of peptides and proteins (36) have allowed this technique to play a pivotal role in proteomics (36–38). Mass spectrometry and proteomics are clearly the approaches of choice for the structural identification of proteasome subunits. They were first used to identify rat liver 26 S proteasome subunits (27). More recently, proteomics was used to study the 20 S proteasome subunits from Trypanosoma brucei (39), Saccharomyces cerevisiae (40), and rat hepatocytes (41) and to identify proteins associated to 20 S proteasomes (42, 43). In addition to protein identification, the ability of mass spectrometry to generate peptide sequence information can be efficiently applied to the determination and the localization of post-translational modifications in proteins. Among these post-translational modifications, phosphorylation has always represented a challenge because of its important role in regulating biological processes, and various strategies based on mass spectrometry have been developed to characterize phosphorylated proteins (44–46). To our knowledge, direct characterization of 20 S proteasome phosphorylation sites using mass spectrometric approaches has never been reported to date.

In this paper, we report on the complete and fine structural identification of all subunits of the human 20 S proteasome, purified from erythrocytes, by a combination of 2D gel electrophoresis, mass spectrometry, and data base search. This allowed us to establish and propose a first so-called “standard reference map” of the human 20 S proteasome that is usable for studies on structure-function relationships. In addition, we successfully used mass spectrometric approaches for the structural identification and the fine analysis of human 20 S proteasome subunit post-translational modifications. In particular, we characterized the N-acetylation on the a7 subunit and localized for the first time its phosphorylation site.

The abbreviations used are: 2D, two-dimensional; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; ACN, acetonitrile; IMAC, immobilized metal ion affinity chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PSD, postsource decay; HPLC, high pressure liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry.

MATERIALS AND METHODS

20 S Proteasome Purification—The proteasome was purified from human erythrocytes by affinity chromatography with the monoclonal antibody MCP21 (European Collection of Cell Cultures) directed against the human u2 subunit as described previously (47). About 8 mg of MCP21 antibody were immobilized on 1 g of CNBr-activated Sepharose (Amersham Biosciences). One hundred ml of human blood from healthy volunteers were mixed with 100 ml of 2% dextran T500 (Amersham Biosciences) in phosphate-buffered saline to sediment the erythrocytes. The pellet was washed five times with cold phosphate-buffered saline, and ¼ volume of water was added. After breakdown by four cycles of freezing/thawing in liquid N2 and at 4 °C, the lysate was centrifuged for 30 min at 20,000 × g to remove cellular debris. The supernatant was incubated overnight at 4 °C with rotary shaking with the MCP21-Sepharose. After washing with 20 mM Tris-HCl, 150 mM NaCl, pH 7.6, the 20 S proteasome was eluted with 20 mM Tris-HCl, 2 mM NaCl, pH 7.6 and then dialyzed for 48 h against 20 mM Tris-HCl, 150 mM NaCl, pH 7.6. Each purification step was performed at 4 °C in the presence of 1 mM EDTA. Homogeneity of the preparation was assayed by native PAGE using a Phast-System (Amersham Biosciences) according to the manufacturer’s instructions. Protein concentration was determined using the Bio-Rad Protein Assay. About 2 mg of 20 S proteasome were purified from 100 ml of blood.

2D Gel Electrophoresis—20 S proteasome preparations were first desalted and concentrated by trichloroacetic acid or ethanol precipitation. The pellet was resuspended in the rehydration buffer (9 mM urea, 2.2% CHAPS, 65 mM dithiothreitol, 0.3% Immobiline pH gradient (IPG) buffer, pH 3–10 NL (non-linear) (Amersham Biosciences)). The first dimension was performed on the IPGphor system. Briefly, the 18-cm pH 3–10 NL IPG strip (Amersham Biosciences) was rehydrated with sample buffer for 11 h under 50 V at 20 °C. Then the sample was focused for 30 min at 200 V, 30 min at 500 V, and 1 h at 2000 V followed by 8000 V for a total of 90 kV-h (whatever the loading amount).

Immediately prior to the second dimension, the IPG strip was incubated in 5 ml of 50 mM Tris-HCl, 6 mM urea, 2% SDS, 30% glycerol, and 65 mM dithiothreitol with rotary shaking for 15 min followed by 15 min in the same solution in which dithiothreitol was replaced by 2.5% iodoacetamide.

SDS-PAGE was performed on the MultiPhor II system using a 12.5% acrylamide precast gel (Amersham Biosciences). Complete migration was achieved at 15 °C after 1 h at 200 V followed by 600 V for a total of 600 V-h. Spots were visualized by Coomassie Brilliant Blue staining.

In-gel Protein Digestion—Stained protein spots were excised and washed twice with milli-Q water. Destaining was achieved by addition of acetonitrile (ACN), rehydration in 0.1 mM NH4HCO3, addition of an equivalent volume of ACN, and drying of the gel pieces in a vacuum centrifuge.

Gel pieces were rehydrated in 12.5 ng/μl trypsin (Sigma) in 50 mM NH4HCO3 and incubated for 5 h at 37 °C. The supernatant was removed and stored at −20 °C, and the gel pieces were incubated overnight in 50 mM NH4HCO3 at room temperature. This second supernatant was pooled with the previous one, and a water/ACN/ HCOOH (50:50:5) solution was added onto the gel pieces for 15 min. This step was repeated again twice. Supernatants were pooled and concentrated in a vacuum centrifuge to a final volume of 10 μl. Digests were concentrated and desalted using a ZipTipC18 (Millipore) nanoscale reverse phase column according to the manufacturer’s instructions.

Phosphopeptide Enrichment by Nanoscale Fe(III)-immobilized Metal Ion Affinity Chromatography (IMAC)—The Fe3+-IMAC column (Millipore) was prepared according to the manufacturer’s instructions.
Briefly, the ZipTip-C18 was equilibrated in H2O/ACN/AcOH (50:50:0.1, v/v/v). The metal ion solution (200 mM FeCl3 prepared in 10 mM HCl) was loaded onto the column by aspirating and dispensing the solution 10 times. The column was washed successively with water, H2O/ACN/AcOH (90:10:0.1, v/v/v), and H2O/ACN/AcOH (90:10:0.1, v/v/v). After concentration to a volume of 5 μl in a vacuum centrifuge and dilution in 20 μl of H2O/ACN/AcOH (90:10:0.1, v/v/v), trypsic peptides were loaded onto the column by aspirating and dispensing the solution 20 times. The column was washed with H2O/ACN/AcOH (70:30:0.1, v/v/v) to remove nonspecific bound peptides. After a last wash in water, phosphopeptides were eluted in 5 μl of 2% NH4OH.

MALDI-TOF Mass Spectrometry Analysis and Data Base Search—Mass spectra were recorded in positive reflectron mode on a MALDI-TOF mass spectrometer (Voyager, DE STR; PerSeptive Biosystems, Framingham, MA) equipped with a delayed extraction device. Mass spectra were recorded in positive reflectron mode on a MALDI-TOF mass spectrometer (Voyager, DE STR; PerSeptive Biosystems, Framingham, MA) equipped with a delayed extraction device. Mass mapping spectra were recorded using α-cyano-4-hydroxycinnamic acid (Sigma) as a matrix (8 mg/ml, H2O/ACN/TFA, 50:50:0.1). A volume of 0.5 μl was mixed on the MALDI target plate with an equivalent volume of sample. IMAC eluates were directly applied onto the plate and were allowed to dry before addition of dihydroxybenzoic acid (Aldrich) matrix. Mass spectra were acquired from m/z 700 to m/z 3000 using the following parameters: 20-kV accelerating voltage, 75% grid voltage, 0.002% guide wire voltage, and 180-ns delay time. The ZipTip-C18 eluate mass spectra were calibrated using autodigestion peaks of trypsin ([M + H]+, 842.51, 2211.10, and 2283.18), while IMAC eluate mass spectra were calibrated by a close calibration to the tryptic peptides ([M + H]+, 2465.20). The postsource decay (PSD) experiment was performed in the reflectron mode with an accelerating voltage of 20 kV, a delay time of 100 ns, and a grid voltage of 80%. The peptide mass fingerprint was submitted to the Protein Prospector software. Peptide masses were compared with the theoretical masses derived from protein sequences contained in either Swiss-Prot or the National Center for Biotechnology Information (NCBI) data bases. Searches were performed using the following parameters: cysteine residues were S-carbamidomethylated, methionine could be oxidized, species were restricted to human, pl was considered in the range 3–10, and molecular mass range was from 103 to 105 Da. Mass tolerance was set to 20 ppm.

On-line Capillary HPLC Nanospray Ion Trap MS/MS Analyses—After MALDI-TOF analyses, peptide extracts were diluted with 10 μl of 0.1% formic acid and analyzed by on-line capillary HPLC (LC Packings) coupled to a nanospray LCO ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). Peptides were separated on a 75-μm inner diameter × 15-cm C18 PepMap™ column (LC Packings). The flow rate was set at 150 nL/min. Peptides were eluted using a 0–40% linear gradient of solvent B in 40 min (solvent A was 0.1% formic acid in 1% acetonitrile, and solvent B was 0.1% formic acid in 90% acetonitrile). The mass spectrometer was operated in positive ion mode at a 2.2-kV needle voltage and a 30-V capillary voltage. Data acquisition was performed in a data-dependent mode consisting of, alternatively in a single run, a full scan MS over the range m/z 370–2000 and a full scan MS/MS in an exclusion dynamic mode. MS/MS data were acquired using a 3 m/z units ion isolation window, a 35% relative collision energy, and a 0.5 min dynamic exclusion duration.

RESULTS

Delineation of the Optimal Conditions for 2D Gel Electrophoresis of Human 20 S Proteasome

In a first step, optimization of the isoelectric focusing conditions was performed to improve the resolution of the 2D gel separation in the first dimension and to obtain the complete separation of all 20 S proteasome subunits. According to the theoretical pl of the 20 S proteasome subunits ranging from 4.8 to 8.7 (Table I) and to maximize the subunit separation, 18-cm non-linear pH 3–10 gradient strips were chosen. To overcome the lack of resolution due to the presence of salts in the 20 S proteasome preparation, a precipitation step was added. Trichloroacetic acid and ethanol precipitations were used. Both allowed the separation of the previously unresolved basic subunits and gave an identical subunit pattern. Ethanol was then selected for further experiments to avoid any possible degradation or modification due to the acidic properties of trichloroacetic acid. Finally, the focusing parameter was also found to be critical for optimal resolution. Best results were obtained for focusing up to 90 kV-h.

Fig. 1 shows the 2D gel pattern obtained with 40 μg of purified material from erythrocytes following the optimized procedure. After staining with Coomassie Brilliant Blue, a total of at least 32 well separated major and minor spots were observed in a range of pl values from 4.8 to 8.6 and of molecular masses from 23 to 32 kDa. This pattern reveals a much higher number of spots than the one of 14 theoretically expected from the 7α and 7β subunits. This observation clearly suggests that the human 20 S proteasome presents a certain degree of complexity and heterogeneity in its subunit composition. When 10 μg of material was loaded onto the gel, minor spots were not detected using Coomassie Brilliant Blue staining. When 80 or 160 μg were loaded, an overall increase in spot intensity was observed, but no additional spot was detected (not shown). Using the optimized procedure, the 2D gel patterns observed were highly reproducible.

Mass Spectrometric Identification of Human Erythrocyte 20 S Proteasome Subunits

Each spot detected on the 2D gel was cut out, subjected to an “in-gel” trypsin digestion, and analyzed by MALDI-TOF mass spectrometry. Each peptide mass mapping obtained was then used to search data bases. This allowed the identification of all labeled spots on Fig. 1. The results, listed in Table I, were obtained from various amounts (10, 40, and 160 μg) of purified 20 S proteasome loaded onto 2D gels. With sequence coverages varying between 13 and 53% depending on the subunit sequence and on the amount of 20 S proteasome used for the analysis, MALDI-TOF mass mapping led to the unambiguous identification of all the spots. The use of further MS/MS experiments was not required at this stage.

As shown in Table I, all 14 of the expected 20 S proteasome subunits were identified. Observed pl and molecular mass values (Fig. 1) were in agreement with the expected theoretical values for most subunits (Table I). Several spots resulted in the same subunit identification after data base search, indicating the presence of numerous isoforms. Detection of these isoforms was reproducible and was observed in 20 S proteasomes purified from all healthy donors, suggesting their natural presence in human erythrocytes under “normal” phys-
Proteomics of Human 20 S Proteasome

TABLE I
Subunit identification of human 20 S proteasome purified from erythrocytes

Protein spots were excised from the 2D gel shown in Fig. 1 and analyzed by MALDI-TOF mass spectrometry. The resulting mass mappings were searched against Swiss-Prot/TrEMBL or against NCBI databases using the Protein Prospector search engine. Correspondence to the older α and β subunit nomenclature is given in parentheses. Theoretical molecular mass and pI values were calculated using the MWCALC algorithm from the Swiss-Prot sequences without propeptide sequences (65). Experimental molecular masses were estimated using the experimental calibration curve generated from the \( R_f \) of low molecular mass markers. Experimental pI values were estimated using the calibration curve supplied for 18-cm pH 3–10 Non-linear IEP strips (Amersham Biosciences). Sequence coverages were calculated from these truncated forms (number of amino acids covered by the identified peptides divided by the total number of amino acids in the protein sequence).

| Spot number | 20 S subunit | Accession number | Experimental molecular mass | Theoretical molecular mass | Experimental pI | Theoretical pI | Sequence Coverage |
|-------------|--------------|------------------|-----------------------------|---------------------------|----------------|----------------|------------------|
| 1           | α5 (i)       | P28066           | 27                          | 26,469                    | 4.8            | 4.86           | 26^a             |
| 2           | β1 (Y)       | P28072           | 23                          | 21,862                    | 5.0            | 5.15           | 34^b             |
| 2'          | β1 (Y)       | P28072           | 23                          | 21,862                    | 4.9            | 5.15           | 13^b             |
| 3           | α7 (C8)      | P25788           | 29                          | 28,302                    | 5.2            | 5.43           | 48^e             |
| 3'          | α7 (C8)      | P25788           | 29                          | 28,302                    | 5.1            | 5.43           | 31^b             |
| 3''         | α7 (C8)      | P25788           | 29                          | 28,302                    | 5.3            | 5.43           | 33^c             |
| 4           | β2 (Z)       | Q99436           | 29                          | 25,295                    | 5.8            | 6.09           | 36^c             |
| 5           | β7 (N3)      | P28070           | 25                          | 24,379                    | 5.6            | 5.76           | 18^a             |
| 5'          | β7 (N3)      | P28070           | 25                          | 24,379                    | 5.4            | 5.76           | 46^c             |
| 5''         | β7 (N3)      | P28070           | 25                          | 24,379                    | 5.9            | 5.76           | 29^c             |
| 6           | α6 (C2)      | P25786           | 32                          | 29,556/30,108^d           | 6.2            | 6.90/7.28      | 25^a             |
| 6'          | α6 (C2)      | P25786           | 32                          | 29,556/30,108^d           | 6.1            | 6.90/7.28      | 18^a             |
| 6''         | α6 (C2)      | P25786           | 32                          | 29,556/30,108^d           | 6.0            | 6.90/7.28      | 30^c             |
| 7           | α1 (i)       | P34062           | 28                          | 27,399                    | 6.2            | 7.01           | 34^a             |
| 7'          | α1 (i)       | P34062           | 28                          | 27,399                    | 6.0            | 7.01           | 24^a             |
| 8           | β3 (C10)     | P49720           | 25                          | 22,930                    | 6.0            | 6.81           | 32^a             |
| 9           | α3 (C9)      | P25789           | 31                          | 29,352                    | 6.7            | 7.95           | 25^a             |
| 9'          | α3 (C9)      | P25789           | 31                          | 29,483                    | 6.2            | 7.95           | 25^a             |
| 9''         | α3 (C9)      | P25789           | 31                          | 29,483                    | 7.3            | 7.95           | 28^a             |
| 10          | α2 (C3)      | P25787           | 26                          | 25,767                    | 6.7            | 7.74           | 27^a             |
| 10'         | α2 (C3)      | P25787           | 26                          | 25,767                    | 6.1            | 7.74           | 36^a             |
| 11          | β4 (C7)      | P49721           | 23                          | 22,836                    | 6.5            | 7.31           | 40^c             |
| 11'         | β4 (C7)      | P49721           | 23                          | 22,836                    | 6.4            | 7.31           | 29^c             |
| 11''        | β4 (C7)      | P49721           | 23                          | 22,836                    | 6.2            | 7.31           | 46^c             |
| 12          | α4 (C6)      | O14818           | 30                          | 27,887                    | 8.2            | 8.67           | 30^c             |
| 12'         | α4 (C6)      | O14818           | 30                          | 27,887                    | 7.8            | 8.67           | 33^c             |
| 12''        | α4 (C6)      | O14818           | 30                          | 27,887                    | 8.6            | 8.67           | 41^b             |
| 13          | β6 (C5)      | P20618           | 25                          | 23,548                    | 7.8            | 8.38           | 40^a             |
| 13'         | β6 (C5)      | P20618           | 25                          | 23,548                    | 7.3            | 8.38           | 53^b             |
| 14          | β5 (X)       | P28074           | 23                          | 22,458                    | 8.2            | 8.66           | 25^a             |

^a Protein identification was performed from gels loaded with 10 μg of purified material.
^b Protein identification was performed from gels loaded with 40 μg of purified material.
^c Protein identification was performed from gels loaded with 160 μg of purified material.
^d Long and short isoform of the α6 subunit.

The majority (12 of 14) of the subunits exhibited multiple (from 2 to 4) isoforms. Only two subunits (α5 and β3) appeared as one detectable spot. As shown by their position on the 2D gel (Fig. 1), the related isoforms of most subunits exhibited clearly different pI values without significant molecular mass variations. The observation that the subunit isoforms were mostly characterized by pI shifts suggested that these isoforms are more likely the result of subunit post-translational modifications rather than the consequence of proteolytic cleavages, mRNA splicing events (49), or artifactual spots arising from the derivatization of cysteine residues with gel components (50).

Mass Spectrometric Characterization of Post-translational Modifications of the Human 20 S Proteasome α7 Subunit

The post-translational modifications occurring at the α7 subunit of the 20 S proteasome are the most documented in the literature. We therefore chose to focus our efforts on this...
subunit to apply mass spectrometric approaches to the structural study of post-translational modifications of the human 20 S proteasome.

All Three /H9251 7 Subunit Isoforms of the Human 20 S Proteasome Are N-Acetylated—After digestion with trypsin, MALDI-TOF analyses were performed on the three isoforms of /H9251 7 (corresponding to spots 3, 3', 3''). The mass spectrum of the tryptic digest obtained from spot 3 is shown in Fig. 2. A total of 14 peptide masses matched the /H9251 7 subunit sequence entry for an amino acid sequence coverage of 48% (Table II). All labeled peaks were attributed to /H9251 7 subunit tryptic peptides and, as usually observed, to trypsin autodigestion peptides. Interestingly, the intense peak at m/z 1959.9 did not match any unmodified sequence of the /H9251 7 subunit but was found as its N-acetylated terminal peptide. We then performed MALDI PSD analysis of the ion at m/z 1959.9 to obtain further sequence information. The MS/MS spectrum (Fig. 3) allowed the unambiguous identification of the N-terminal peptide sequence of /H9251 7 bearing an acetylated N terminus. This acetylation was detected in all three of the /H9251 7 isoforms, therefore excluding this post-translational modification as the cause of the pI shifts observed for spots 3, 3', and 3'' on the 2D gel.

Two of the Three /H9251 7 Subunit Isoforms of the Human 20 S Proteasome Are Phosphorylated—MALDI-TOF analyses of the three /H9251 7 isoform tryptic digests gave a similar peptide mapping, although the number of peptides detected varied depending on the protein quantities analyzed. To detect a possible phosphorylation, the modification was taken into consideration for data base search, but no phosphopeptide was found. This may be explained by the poor ionization yield of phosphopeptides in MALDI-TOF analysis when present in complex peptide mixtures. We then performed phosphopeptide enrichment by IMAC using an iminodiacetic acid matrix and Fe$^{3+}$ ions. MALDI-TOF mass spectra of the /H9251 7 subunit digests from spots 3, 3', and 3'' after phosphopeptide enrichment are presented in Fig. 4, A–C, respectively. The mass spectrum of the most intense isofrom tryptic digest (spot 3) showed two major peaks at m/z 1735.6 and m/z 1751.6. The lower mass (m/z 1735.6) corresponded to the C-terminal peptide sequence of the /H9251 7 subunit, 242ESLKEEDESDDNM 255, bearing one phosphorylation group. The higher mass (m/z 1751.6) corresponded to the same phosphorylated peptide with an oxidized methionine residue. These two phosphorylated peptides were also observed in the /H9251 7 tryptic digest from the weaker spot 3' at a more acidic pI (Fig. 4B) but not in the digest from spot 3' at a less acidic pI for which two peaks at

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**Fig. 1.** Two-dimensional electrophoretic reference map of human 20 S proteasome. 20 S proteasome from erythrocytes (40 μg) was separated using a pH 3–10 non-linear IPG strip in the first dimension followed by a 12.5% SDS gel in the second dimension. The 2D gel was stained with Coomassie Brilliant Blue. All labeled spots were identified by MALDI-TOF mass spectrometry and data base search (see Table I).

**Fig. 2.** MALDI-TOF peptide mass mapping of the /H9251 7 subunit tryptic digest from spot 3. The mass spectrum was calibrated using autodigestion peaks of trypsin (T: m/z 842.51, 2211.10, and 2283.16). Taking into account the N-acetylation of the protein, all other labeled peaks matched /H9251 7 tryptic peptide sequences (see Table II) as a result of data base (Swiss-Prot) search, reaching a 48% sequence coverage.
m/z 1655.6 and m/z 1671.6 were detected instead (Fig. 4C). In this case, the measured masses corresponded to the same C-terminal peptide of the α7 subunit, \textit{\textsuperscript{242}ESLKEEDESDDDNM\textsuperscript{255}}, without any phosphorylation (m/z 1655.6) and to its oxidized analogue (m/z 1671.6). The detection of these non-phosphorylated peptides, although after the IMAC procedure, could be explained by the presence of acidic residues in the peptide sequence. Such residues are known to be able to bind to the IMAC column as exemplified by the intense peak detected at m/z 989.5, which corresponds to the acidic \textit{\textsuperscript{231}DIREEAEK\textsuperscript{238}}.

The Two α7 Subunit Isoforms of the Human 20 S Proteasome Are Phosphorylated on a Unique Residue, Ser\textsuperscript{250}—Since the identified C-terminal peptide contained two possi-

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**Table II**

Identification of the α7 subunit by MALDI-TOF mass mapping and data base search

Tryptic peptide masses (expressed in Da) determined by MALDI-TOF mass mapping of spot 3 (Fig. 2) are compared to the theoretical values resulting from the data base search. Corresponding peptide sequences, positions in the protein sequence, and amino acid modifications are indicated. Met-ox, oxidized methionine residue; N-Ac, N-acetylation.

| Submitted mass [M + H]\textsuperscript{+} | Theoretical mass [M + H]\textsuperscript{+} | Sequence | Position | Modification |
|------------------------------------------|------------------------------------------|----------|----------|-------------|
| 722.40                                   | 722.42                                   | HEIVPK   | 225–230  |             |
| 745.42                                   | 745.42                                   | SLADIAR  | 87–93    |             |
| 763.41                                   | 763.41                                   | LFNVDR   | 67–72    |             |
| 989.49                                   | 989.49                                   | DIREEAEK | 231–238  |             |
| 1095.55                                  | 1095.54                                  | LYEEGSNK | 58–66    |             |
| 1114.56                                  | 1114.56                                  | VFQVEYAMK| 21–29    |             |
| 1152.61                                  | 1152.60                                  | SNFGYNPLK| 101–110  |             |
| 1217.65                                  | 1217.65                                  | AVENSSTAIGIR | 30–41 |             |
| 1228.71                                  | 1228.69                                  | IYIVHDEVK| 197–206  |             |
| 1237.63                                  | 1237.62                                  | CKDGVVFVEK| 42–52    |             |
| 1380.76                                  | 1380.74                                  | HVGMAVAQLADAR | 73–86 |             |
| 1396.75                                  | 1396.73                                  | HVGMAVAQLADAR | 73–86 | Met-ox    |
| 1471.83                                  | 1471.81                                  | IYIVHDEVKDK | 197–208  |             |
| 1959.91                                  | 1959.89                                  | SSIGTGYDLSASTFSPDGR | 2–20 | N-Ac      |

**Fig. 3.** Characterization of the N-acetylation of the α7 subunit by postsource decay MALDI-TOF mass spectrometry. The PSD spectrum of the acetylated peptide “SSIGTGYDLSASTFSPDGR\textsuperscript{20} (M + H)\textsuperscript{+} at m/z 1959.9) from spot 3 (Fig. 2) displays b- and y-type fragment ions and dehydrated b ions (labeled as b*) according to Biemann’s nomenclature (64). The fragmentation pattern unambiguously identifies the N-terminal acetylation of the α7 subunit.
ble sites of phosphorylation (Ser243 and Ser250), we performed MALDI PSD analysis of the phosphorylated peptide from spot 3 at m/z 1735.6 (Fig. 5). These experiments revealed that none of the observed b-type fragment ions, which include the Ser243 residue but not the Ser250 residue, was phosphorylated. This allowed us to locate unambiguously the phosphorylation site on the Ser250 residue. We confirmed this result by MS/MS analyses using a nanospray ion trap mass spectrometer, which allowed the detection of a more complete series of b-type fragment ions (Fig. 6A). These analyses further allowed the localization of the phosphorylation site on the less intense phosphorylated peptide from spot 3/H11032. The MS/MS spectrum clearly showed that the phosphorylation group was localized at the same site, i.e., on the Ser250 residue (Fig. 6B).

**DISCUSSION**

In this study, proteomics based on 2D gel electrophoresis and mass spectrometric identification was used to establish the complete and detailed reference map of human 20 S proteasome. Erythrocytes from healthy individuals represented an abundant and reproducible source of human 20 S proteasome, allowing us to obtain the amount of purified material necessary to perform unambiguous structural characterization in addition to subunit identification. Moreover, 20 S proteasome from erythrocytes represented a rather homogeneous material since erythrocytes possess only cytoplasmic standard proteasome. It can thus be considered as a simple model and appropriate reference for human 20 S proteasome.

In our study, optimization of the 2D gel electrophoresis conditions was achieved using non-linear IPG strips, which increased the resolution of the isoelectric focusing separation and its reproducibility (39, 51–53). The 14 α and β subunits of human 20 S proteasome were then unambiguously identified by MALDI-TOF mass mapping of corresponding tryptic peptide digests. Their location on the 2D gel were in good agree-
ment with the corresponding theoretical pI and molecular mass values. However, an important observation was the presence of multiple isoforms for most of the subunits, resulting in a total of at least 32 visible spots after Coomassie Blue staining. Multiple isoforms of eukaryotic proteasomes have already been reported in rat liver (54) and Drosophila (13). In human, 2D gel electrophoresis combined with detection and identification techniques mainly based on Western blotting and/or Edman degradation has been applied to study 20 S proteasome composition from erythrocytes, tissues (placenta, kidney, liver), and cell lines (14, 15, 48, 55). These studies also revealed the presence of different isoforms for most of the human 20 S proteasome subunits. Thus, our data not only support but also complement these previous observations by providing evidence for the first time that in human this structural heterogeneity not only exists for $\alpha$ subunits but also within $\beta$ subunits, including the catalytic subunits. As compared with the experimental conditions used in earlier studies, the use of the more resolutive and sensitive 2D gel electrophoresis tools currently available (such as non-linear pH gradient strips) contributed to the ability to obtain the fine reference map of the human 20 S proteasome presented here. The fact that the observed isoforms of a given subunit display shifted pI values rather than different molecular masses suggests that their presence is more likely the result of post-translational modifications rather than proteolytic cleavages. The human 20 S proteasome thus exhibits a high degree of structural heterogeneity, which could account for variations in catalytic activities observed for proteasome subtypes (12).

In association with separative techniques, such as 2D gel electrophoresis and HPLC, and with bioinformatics tools, mass spectrometry is a core technique of proteomics. Indeed, not only do the sensitivity and resolution achieved with nanospray and MALDI mass spectrometers allow the identification of proteins in complex mixtures, but the ability of mass spectrometry to generate peptide sequence information can also be efficiently applied to the determination and the localization of post-translational modifications in proteins. One of the aims of our study was thus to develop such an approach. Among these post-translational modifications, phosphorylations remain the most challenging ones since they play an important role in regulating biological processes. Various strategies based on mass spectrometry have been developed to characterize phosphorylated proteins (44–46). Difficulties in analyzing phosphopeptides by mass spectrometry arise from their often low abundance and from their low ionization yield, particularly in MALDI experiments, when they are present in complex peptide mixtures. Phosphopeptide enrichment using IMAC prior to mass spectrometric analyses has proven to be an efficient strategy to analyze phosphopeptides in complex mixtures (45, 56, 57). Many studies have thus been conducted to improve binding and elution conditions of these phosphopeptides (58–60) and to find an elution buffer compatible with mass spectrometry. However, to our knowledge, no data have been reported yet on the use of mass spectrometry for the characterization of phosphorylation sites of proteasome subunits.

To validate the use of mass spectrometric approaches as a mean for a precise structural dissection of post-translational modifications with the corresponding theoretical pI and molecular mass values, localization of the phosphorylation site of the $\alpha$7 subunit from spot 3 by postsource decay MALDI-TOF mass spectrometry. The PSD spectrum of the monophosphorylated peptide $^{242}$ESLKEEDE$^{255}$SDDDDN[M + H]$^+$ at m/z 1735.6 from spot 3 (Fig. 4) displays b- and dehydrated b-type fragment ion series according to Biemann’s nomenclature (64). The fragmentation pattern is consistent with the phosphorylation of the Ser$^{250}$ residue (but not the Ser$^{243}$ residue) as indicated by the circled P on the peptide sequence. Intense loss of phosphoric acid ($\text{H}_3\text{PO}_4$, 98 Da) by $\beta$-elimination from the precursor peptide ion is indicated on the spectrum by a circled $\beta$.
modifications of the human 20 S proteasome, we chose to focus our efforts primarily on the α7 subunit. Indeed, phosphorylation of 20 S proteasome subunits, which contain several consensus sequences for tyrosine and serine/threonine kinases, may vary according to the species and the tissue. Phosphorylation of 20 S proteasomes from rat fibroblasts and human embryonic lung cells (26) and from normal rat kidney cells (28) has been reported. In mammalian cells, phosphorylation on serine residues of α3 and α7 subunits has been described (25, 26). In our study, we only detected phosphorylation on Ser^{250}, not on Ser^{243}, of the human 20 S proteasome α7 subunit, whereas site-directed mutagenesis has shown that both Ser^{243} and Ser^{250} residues could be phosphorylated by casein kinase II in mammalian cells (28). This observation may suggest a different phosphorylation pattern of 20 S proteasome in human erythrocytes as compared with other mammalian tissues.

Considering that failure to detect both phosphorylated
Ser^{250} and Ser^{243}, which are included in the same tryptic peptide sequence, may result from a low efficacy of the experimental procedures we used, i.e. IMAC enrichment followed by MALDI-TOF/MS detection, we performed additional experiments using Ga^{3+} instead of Fe^{3+} as the metal ion in the IMAC experiments. While Fe^{3+} ions are more efficient to bind monophosphorylated peptides, Ga^{3+} ions have been shown to preferentially bind multiphosphorylated peptides (60). However, no additional peptide was observed using Ga^{3+} ions as the chelating agent. To circumvent the possible weakness of IMAC combined with MALDI-TOF/MS detection, we performed capillary HPLC/nanospray-MS/MS experiments on digests from spots 3, 3’, and 3”, but no multiphosphorylated peptide could still be detected. These additional experiments further supported the absence of phosphorylation on Ser^{243} of the α7 subunit of human erythrocyte 20 S proteasome. The functional and/or physiological significance of a specific phosphorylation pattern of the human erythrocyte proteasome remains to be established.

Beside phosphorylation, N-acetylation seems also to be another structural feature commonly observed in erythrocytes (55), rat liver (21), and yeast (22, 23, 61) for proteasome subunits other than the catalytically active β subunits of which the N-terminal active residue is protected by a propeptide. In our study, N-acetylation of a human proteasome subunit was evidenced by mass spectrometric approaches. Using both MS and MS/MS experiments, we were able to detect an N-acetylated tryptic peptide corresponding to the N-terminal sequence of the α7 subunit. However, no other N-acetylation was found using MALDI/MS experiments. The suppression effect observed when analyzing complex peptide mixtures makes the analysis of the complete sequence of a given protein difficult using a single approach.

In conclusion, the activity and functionality of the human 20 S proteasome are tightly linked to its subunit composition and structural properties. In addition to the known standard proteasome and immunoproteasome, other proteasome subtypes have been found to differ in their catalytic activities (12). Also, changes in proteasome composition has been evidenced in vivo during Drosophila development (13), B cell differentiation (62), goldfish ovocyte cell cycle (63), and rat coronary occlusion/perfusion (24). The perfect knowledge of the structural modifications affecting the proteasome subunits is thus a key element to understand its function or dysfunction under normal or pathological conditions.

The precise identification of post-translational modifications of proteasome may be achieved using mass spectrometric approaches and will benefit from the constant evolution of these technologies. It will take great advantage from recent and improved MS approaches such as nanospray ionization, which reduces suppression effects, combined with ion trap analyzers or hybrid Q-TOF, which generate more informative MS/MS spectra. In addition, the capability to determine the precise molecular mass of each isoform of a given subunit will represent an important improvement. In particular, it will allow a comparative analysis between measured and theoretical molecular masses of the subunits and will thus give information about the nature and the number of post-translational modifications affecting a proteasome subunit. Experimental developments are currently in progress in our laboratory to reach this objective.

* This work was supported in part by grants from the CNRS (Action Concertée Inititative program Protéomique et Génie des Protéines), the Association pour la Recherche contre le Cancer (Contrat N 4435 Réseau Alliance des Recherches sur le Cancer, Protéomique et Cancer), the Conseil Régional Midi-Pyrénées, and the Consortium Pierre Fabre Médicament (Program Après Sénquencé Génomique, Protéomique et nouvelles cibles thérapeutiques). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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