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20S Human proteasomes bind with a specific orientation to lipid monolayers in vitro

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Abstract

20S Proteasomes are non-lysosomal, high molecular weight proteinases implicated in the degradation of misfolded proteins and several short-lived regulatory proteins. They have a well established role, as the core of the 26S proteasome complex, in the ubiquitin-dependent proteolytic pathway and in antigen processing. While correctly folded proteins are not degraded by the 20S proteasome, unfolding, for example by oxidation, may render them degradable. The 20S proteasome is a 700-kDa cylindrical particle, composed of 14 subunits of molecular masses 20–35 kDa. There is evidence that 20S proteasomes are in close proximity to or associate with the endoplasmic reticulum and nuclear and plasma membranes in vivo. To better understand the lipid association of 20S proteasomes in vitro, we used a lipid monolayer system as a simple model system for biological membranes. The structure and orientation of the monolayer lipid bound 20S proteasomes has been determined by electron microscopy. 20S proteasomes associated in an 'end-on' configuration specifically on PI lipid monolayers forming large arrays, with their channels opposite the lipid headgroups. On ER and Golgi lipid films 20S proteasomes were oriented in the same way as on the PI lipid film but were monodisperse. Protein molecules were randomly oriented in the presence of PA, PG, PS, PC and mitochondrial lipid monolayers. We show that 20S proteasomes bind to phospholipids in vitro in a preferred orientation which places the proteasome channel perpendicular to the membrane.

Keywords: Proteasome, 20S; Lipid monolayer; Electron microscopy

1. Introduction

20S Proteasomes are 700 K multicatalytic proteinases, found in the cytoplasm of many eukaryotic cells [1] and are involved in non-lysosomal degradation of misfolded proteins and several short-lived regulatory proteins. 20S proteasomes contain several proteolytically active sites with different specificities [2] and are able to break a variety of peptide bonds and to fragment substrate peptides to small peptides, possibly by channelling intermediates between different catalytic sites [3,4]. As the proteolytic core of an even larger multifunctional complex, designated the 26S proteasome, the proteasome catalyses the degradation of ubiquitin-protein conjugates [5]. 20S Proteasomes constitute up to 2% of soluble cellular protein [6] and have been localised in the cytoplasm and nucleus [7].

In the present study, 20S proteasomes were isolated from the cytoplasm of Raji B lymphocyte cells and were purified to homogeneity. The 700-kDa polypeptide was isolated by chromatography and sucrose gradient ultracentrifugation. The specificity of interaction of proteasomes with various lipids was investigated using monolayers of different authentic phospholipids and lipids extracted from dog pancreatic microsomes, rat Golgi and mitochondrial preparations [8] and a Langmuir trough [9]. 20S Proteasomes were injected under the preformed lipid monolayers at a surface pressure similar to in vivo lipid conditions [10].

Abbreviations: PI, bovine phosphatidylinositol; ER, endoplasmic reticulum membrane lipids; PA, L-a-dimyristoylphosphatidic acid; PG, L-a-dipalmitoylphosphatidyl-glycerol; PS, bovine phosphatidylserine; PC, bovine L-a-phosphatidylcholine; SLLVY-MCA, succinyl-Leu-Leu-Val-Tyr-4-methyl-7-coumarylamide; AMC, aminomethylcoumarin; DMSO, dimethylsulfoxide; DTT, dithiothreitol; EM, transmission electron microscopy.

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2. Materials and methods

2.1. Purification of 20S proteasomes

20S Proteasomes were purified from packed Raji cells (from a 50-l culture) which were diluted with an equal volume of 20 mM Tris buffer (pH 7.4), containing 20 mM NaCl, 1 mM EDTA and 1 mM DTT (buffer A) and the cells lysed by five passes of the pestle through a Potter homogeniser. The lysate was centrifuged at 80 000 × *g* for 60 min (50.2Ti rotor; Beckman Instruments Inc., Palo Alto, CA). The supernatant was then removed and ammonium sulphate fractionation carried out at 35% saturation by adding 0.205 g ammonium sulphate per ml lysate, with continuous stirring for a further 30–45 min. After centrifugation at 40 000 × *g* for 40 min (50.2Ti rotor) to pellet the precipitate, the supernatant was decanted into a beaker and a further 0.205 g/ml of ammonium sulphate added to bring the final ammonium sulphate concentration up to 60% saturation. After stirring for 60 min the mixture was centrifuged at 40 000 × *g* for 40 min (50.2Ti rotor). Pelleted material was resuspended to a final volume of 50 ml with 20% volume lysis buffer and dialysed overnight against 20 mM Tris buffer (pH 7.4), containing 20 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT and 20% glycerol. The dialysed sample was then loaded onto a DEAE Sepharose column followed by application of a linear gradient of increasing NaCl resulted in the elution of about 1 mg of 20S proteasome at 0.20 M NaCl as determined by Bradford protein assay [11]. The active fractions were collected after assaying against the substrate SLLVY-MCA. The active fractions obtained from the DEAE-Sepharose column were made to 5 mM potassium phosphate (pH 7.6) plus 20% glycerol and loaded onto a hydroxyapatite column (3 cm × 10 cm). The protein was eluted by a 400 ml gradient (5–200 mM potassium phosphate). The most active fractions were collected, dialysed against buffer A and loaded onto a Mono-Q column. The protein was eluted by using a salt gradient (20–500 mM NaCl). Samples (about 10 μg) were subsequently checked for purity by polyacrylamide gel electrophoresis using sodium dodecyl sulphate (SDS-PAGE) under reducing conditions [12], and protein-visualised with Coomassie blue. The protein was dialysed against 20 mM NaCl in 20 mM Tris-HCl buffer (pH 7.5) and concentrated using Centricon microconcentrators (Amicon, MA, USA). Linear sucrose gradients (35 ml) were prepared in a gradient former from stock solutions of 10% (w/v) and 35% (w/v) sucrose in 20 mM Tris, 100 mM NaCl, 0.5 mM dithiothreitol, 0.1 mM EDTA, 5% (v/v) glycerol, 0.02% (w/v) sodium azide buffer at pH 7.6. Partially purified proteasomes (1 ml) from the anion exchange step were applied to gradients cooled at 4°C and subjected to ultracentrifugation using a SW28 rotor (Beckman) at about 140 000 × *g* over 40 h at 4°C. At completion, fractions (1 ml) were collected (0.5 ml/min) from the bottom of the centrifuge tube. Final protein concentrations were determined using the Bradford protein assay and was approx. 6 mg.

2.2. Peptidase activity assay

Peptidase activity was determined for samples of 20S proteasomes by incubating them with 0.25 mM of synthetic substrate SLLVY-MCA plus 100 mM Tris-HCl (pH 8.0) in a final volume of 100 μl. The assays were carried out for 1 h at 37°C. The reaction was terminated by adding 2.6 ml of 100 mM Tris-HCl (pH 9.0) with 0.9% SDS. The release of fluorescent AMC was measured on a Perkin-Elmer luminescence spectrometer (model LS 50B) at 366 and 420 nm for excitation and emission wavelengths, respectively. Assays for proteasome activity against fluorogenic peptides was as for peptidase activity above but with lipids dissolved in DMSO added to the assay mixture.

2.3. Monolayer formation and measurement

Monolayers were formed of purified phospholipids from Avanti (Alabama, USA). Lipids were dissolved in chloroform at a concentration of 25 μg/ml. Lipid monolayers were formed by spreading 1 μl of the solution on a clean 20 mM Tris-HCl (pH 7.5) plus 20 mM or 200 mM NaCl, subphase in a teflon Langmuir trough with a surface area of 50 mm^2. This was followed by compression of the lipid monolayer to the desired initial surface pressure and injection of 10 μl of 350 μg/ml proteasome under the monolayer to give a 3.5 μg/ml final concentration of protein in the subphase. The surface pressure was measured at constant surface area with an automated surface barostat apparatus incorporating a Wilhelmy balance with a platinum plate and transducer (KSV 2200, Finland). The teflon trough was a modified 'zero-order' trough [9] with a total volume of 1.0 ml that was scrupulously cleaned before each experiment using ethanol and double-distilled water. All the measurements were taken at room temperature (22°C). Lipids extracted from dog pancreatic microsomal membranes and Golgi preparations, using the modified Folch-Lees procedure [8], and the phospholipids PI and PC (from Avanti Polar Lipids, Inc.) were each spread to form monolayers with surface pressures of 25 mN/m. The aqueous subphase consisted of 0.1 M Tris-HCl buffer (pH 7.5), containing 20 mM NaCl. 20S Proteasomes were injected through the pre-formed monolayer into the subphase, with continuous stirring.

2.4. Electron microscopy

20S Proteasomes, in the absence of lipid monolayer, were examined in the electron microscope after collection on hydrophilic carbon-coated grids, prepared by 'glow-discharge', and staining with 1% uranyl acetate.

Lipid monolayer/20S proteasome complexes were picked up directly from the Langmuir trough, after incuba-

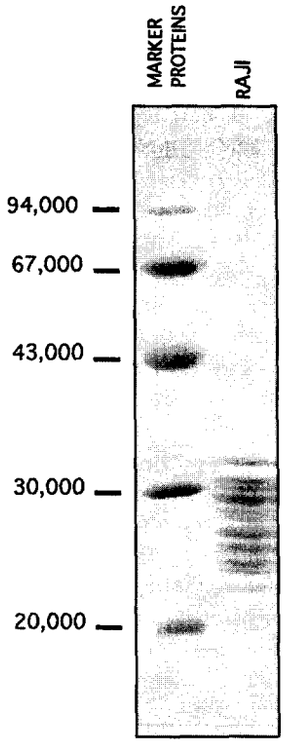

Fig. 1. SDS-PAGE of 5 μg of purified 20S proteasomes from the Raji cell line, lane 2, and of molecular mass markers, lane 1, (12% acrylamide). Molecular mass markers for lane 1: phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase and soybean trypsin inhibitor.

tion at 20°C for 1 h at surface pressures corresponding to the maximum binding as shown in Fig. 3, namely 29 mN/m for PI and 27 mN/m for ER and Golgi lipids. The complexes were picked up using freshly prepared carbon-coated formvar 400 mesh electron microscope grids by

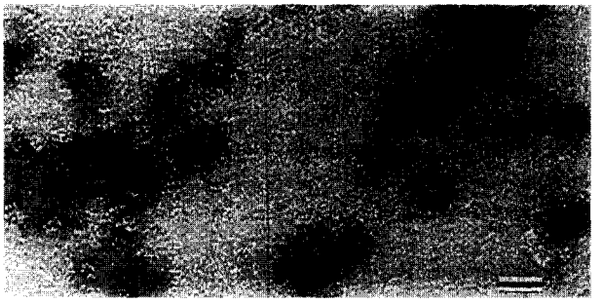

Fig. 2. Negatively stained images of purified 20S proteasomes. Monodisperse particles showing characteristic four stacked ring in side view projections and rings in end-on projections (the scale bar represents 50 nm). The protein molecules were adsorbed onto a carbon-coated formvar grid and negatively stained with 1% uranyl acetate before air-drying. For examination, a Zeiss EM10 electron microscope was used at 80 kV with a 70-μm objective aperture.

touching the grid carbon side down, horizontally on the monolayer, and carefully lifting it off the surface. The grid plus lipid monolayer/20S proteasome complex was touched briefly on the side of a filter paper to remove excess liquid and floated on a drop of 1% (w/v) uranyl acetate for 20 s before air-drying and examination in a Zeiss EM10 transmission electron microscope at an accelerating voltage of 100 kV.

3. Results and discussion

3.1. Electron microscopy of purified 20S proteasomes

The purified 20S proteasome showed the presence of at least ten components with molecular masses ranging from 22–34 kDa by SDS-PAGE (Fig. 1). Analysis of the puri-

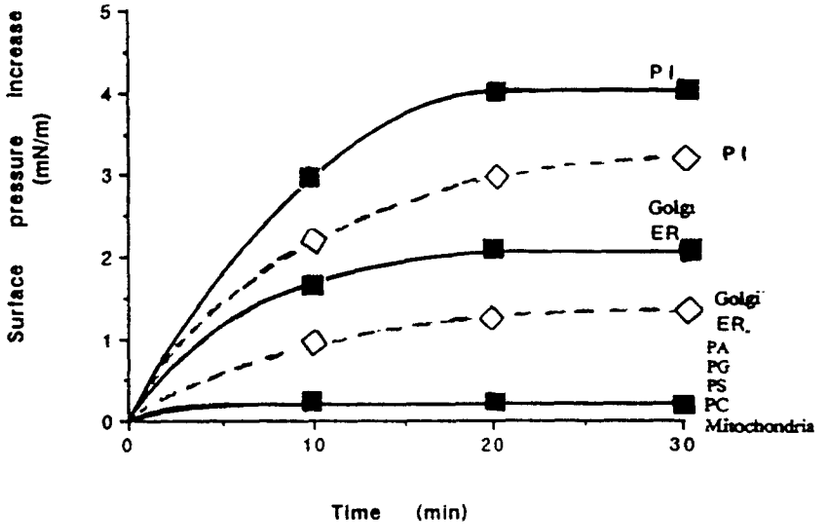

Fig. 3. Increase in surface pressure, relative to the initial surface pressure of monolayer lipid at 25.0 ± 0.1 mN/m, after injection of 350 μg of 20S proteasomes to give a final protein concentration of 3.5 μg/ml. Lipid monolayers used were PA, PG, PS, PC, PI, ER, mitochondrial and Golgi lipids and the buffer was 20 mM Tris-HCl (pH 7.5), containing either 20 mM (solid line) or 200 mM NaCl (dotted line).

fied 20S proteasomes by negative-stain EM revealed characteristic structures 11 nm wide and 15 nm long [13]. 20S Proteasomes, seen in projection, were monodisperse, with the number of particles lying flat greater than those in cross-section (Fig. 2), with four stacked rings composed of 7 subunits, similar to previously described images of 20S human proteasomes [14].

3.2. Interaction with phospholipids

The variation in surface pressure at constant surface area, with a final concentration of 3.5 μg/ml of 20S proteasome in 20 mM or 200 mM salt under seven different kinds of lipid monolayers, is shown in Fig. 3. All curves represent the average of four repeat measurements. It can be seen that there was an increase in surface pressure of 4.0 ± 0.1 mN/m when 20S proteasomes were injected under a PI monolayer at a surface pressure of 25.0 ± 0.1 mN/m. When 20S proteasomes were injected underneath an ER and Golgi lipid monolayers at a pressure of 25.0 ± 0.1 mN/m the increase was 2.0 ± 0.1 mN/m. Using a PC monolayer and mitochondrial lipid monolayer there was a negligible increase in surface pressure after proteasome injection into the subphase at an initial surface pressure of 25 ± 0.1 mN/m. At the higher salt concentration of 200 mM NaCl there was less of an increase in the surface pressure under PI, ER and Golgi lipids, but the increase was still significant (dotted lines). The increase in surface pressure induced by interaction of 20S proteasomes with PI, Golgi and ER lipid was not as great as that observed when a portion of polypeptide inserts into the monolayer. Thus, for example, an increase of 21 mN/m was measured when the gene fusion protein ApoA1/protein A complex at a concentration of 2 nM (compared to 5 nM for the 20S proteasome concentration), penetrated the lipid monolayer by insertion of the ApoA1 component into the hydrophobic acyl region (Newman, unpublished data). Given the much smaller increase in surface pressure induced by 20S proteasomes, it appears that they interact directly with the lipid polar headgroup. The decreased association of 20S proteasomes with lipids at higher salt concentration suggests that the proteasome/lipid monolayer interaction is electrostatic, and also headgroup-specific in that their is no interaction with other acidic lipids (PS and PG). From these results it is clear that, under these conditions, 20S proteasomes interact extensively with PI lipid, less so with ER and Golgi lipids, and to a negligible extent with PA, PG, PS, PC and mitochondrial lipids. In terms of lipid composition, the percentage of PI in ER and Golgi membranes is of the order of 10%, whilst it is of the order of 1% for mitochondria. The order of specificity of interaction of 20S proteasomes with phospholipids has no obvious bearing on 20S proteasome function, as judged by reference to the literature. However, the specific binding of 20S proteasomes to ER and Golgi lipids rather than mitochondrial lipids indicates the possibility of a definite location of the protease in subcellular membranes. This is interesting in view of the observation that proteasomes from rat hepatocytes and cultured human cells, tested with polyclonal antibodies that recognise many of the different subunits, have shown them to be located close to the ER [15]. Although 20S proteasomes were not found associated with the Golgi in this study this could be explained by the different cell lines used, as the distribution of 20S proteasomes between different cell compartments can vary according to the cell type.

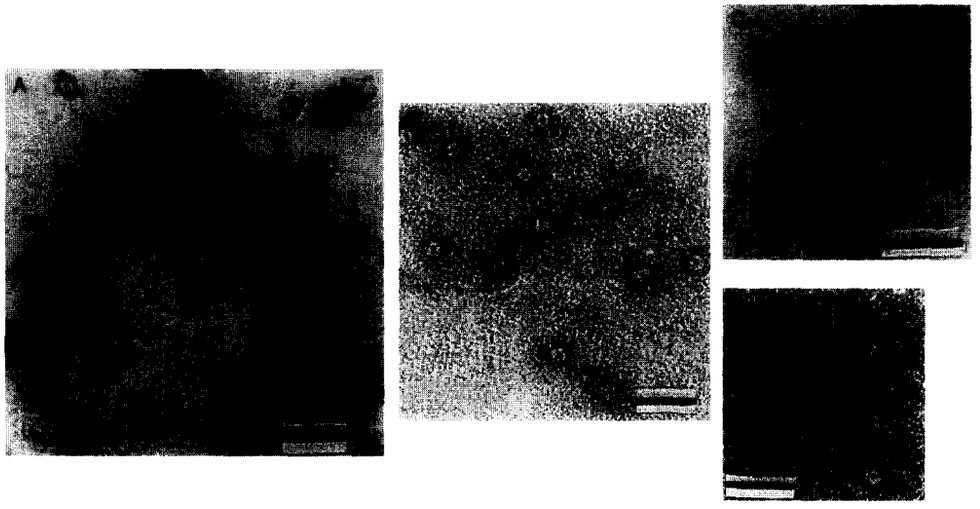

Fig. 4. Electron microscope images of 20S proteasomes in the presence of monolayer lipids. (a) An array of 20S proteasome molecules formed on a monolayer of PI. Ring-like structures about 11 nm in diameter can be seen over most of the field. In places they assemble to form small, regular 'islands'. The scale bar represents 55 nm. (b) 20S proteasomes bound to a monolayer of ER lipid. Monodisperse ring-like structures are scattered throughout the field The scale bar represents 55 nm. (c) 20S proteasomes are scattered in random orientations in the presence of a PC lipid monolayer (the scale bar represents 65 nm) and (d) a mitochondrial lipid monolayer (the scale bar represents 50 nm).

3.3. Electron microscopy of 20S proteasomes on lipid monolayers

Lipid monolayer-20S proteasome complexes examined by electron microscopy showed different 20S proteasome orientations. 20S Proteasomes had assembled in an 'end-on' configuration (Fig. 4A) on PI lipid monolayers forming large arrays, with their channels opposite the lipid headgroups. On ER (Fig. 4B) and Golgi lipid films 20S proteasomes were oriented in the same way as on the PI lipid film but were monodisperse. Protein molecules were randomly oriented in the presence of PC (Fig. 4C) and mitochondrial lipid (Fig. 4D) monolayers, suggesting they did not interact with these lipids consistent with the lipid monolayer interaction results. Native 20S proteasomes were examined after being picked up on hydrophobic grids and showed a random orientation (data not shown). Lipid monolayer films were not present on hydrophilic grids perhaps being destroyed by the application of the grid, or by not being captured. This suggests that there is a specific interaction with PI headgroups, rather than a purely electrostatic interaction with negatively charged lipids. The presence of the lipid monolayer was confirmed by comparison of 20S proteasome/lipid images from hydrophilic and hydrophobic grids. On hydrophilic grids there was no difference in images of proteasome/lipid in terms of orientation and packing from images of proteasomes in the absence of lipid monolayer. This was in contrast to the images obtained from proteasomes/lipid monolayers on hydrophobic grids, confirming that a hydrophobic surface was needed to pick up the lipid monolayer with bound proteasomes.

The atomic structure of the archaebacterial proteasome [16] shows that the α-subunits make up the ends of the cylinder, with the β-subunits forming the middle two rings. The β-subunits have been shown to be the catalytic sites and to have threonine protease activity [4]. In view of the highly conserved quaternary structure of proteasomes, it is probable that the eukaryotic homologues of the α- and β-subunits occupy equivalent positions in the molecule, i.e., the β-subunit homologues constitute the two central rings of the cylinder whilst the α-subunits form the outer rings. In the 26S proteasome, the 20S proteasome is identical to and functions as the proteolytic core of the 26S complex [5], whilst a regulatory 19S protein complex participates in the ATP-dependent degradation of ubiquitinated proteins. Electron microscopical studies have shown that the 26S complex is assembled from the cylindrical 20S particle, to which two large, cap-shaped complexes are bound end-on, resulting in a dumb-bell-shaped complex [17]. Only unfolded proteins can enter the proteolytic core so that the 19S complex serves as the entry point for substrates and stimulates the peptidase activity [18] of the 20S proteasome. Another 20S proteasome activator protein, PA28, a homo-oligomeric ring-like particle, also forms a stable complex by capping at both ends of the 20S proteolytic core [19]. Several other proteins have been identified which may be involved in 20S proteasome regulation and, although not all have been investigated in detail, it is conceivable that these components also regulate the 20S proteasome by capping its ends [20,21]. The 26S complex is unlikely to bind to lipid in the way that we have shown the 20S proteasome does because of the 19S complex which caps the putative lipid binding sites. As purified 20S proteasomes were initially isolated from the cytoplasm of cells in the absence of detergent, the positioning and localisation of 20S proteasomes on ER and Golgi lipids in vitro in the absence of substrate, implies that particles can associate with (PI) lipid-containing membranes.

4. Conclusions

We have established that 20S proteasomes interact with a lipid membrane at the structural level. As part of the specific interaction of the 20S proteasome with monolayers of phosphatidylinositol, Golgi and endoplasmic reticulum lipids, there is an accompanying structural orientation, proteasomes orienting 'end-on' to the headgroups of the lipid monolayer. Further studies are required to establish the in vivo relevance of these specific protein–lipid structural observations.

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