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Localization of subunits in proteasomes from *Thermoplasma acidophilum* by immunoelectron microscopy

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The subunit topography of the *Thermoplasma acidophilum* proteasome was determined by immunoelectron microscopy using monospecific antibodies directed against the two constituent subunits (α,β). Anti-α-subunit IgG was found to bind to the outer disks of the cylinder- or barrel-shaped molecule, while the binding sites of the anti-β-subunit IgG were mapped on the two inner rings. Probably the homologues of the two subunits in the compositionally more complex but isomorphous eukaryotic proteasomes occupy equivalent positions.

Proteasome; Multicatalytic proteinase; Archaebacterium; Immunoelectron microscopy

1. INTRODUCTION

The proteasome is a high molecular weight (ca. 700 kDa), non-lysosomal multicatalytic proteinase, ubiquitous in eukaryotic cells; its properties were recently reviewed by Rivett [1] and Orlowski [2]. Eukaryotic proteasomes have a complex multisubunit structure, characteristically composed of 10–20 different subunits, all in the molecular weight range from 20–35 kDa. The similarity of the currently available sequences indicates that the individual subunits of the proteasome are encoded by members of the same gene family [3,4].

From the archaebacterium *Thermoplasma acidophilum* we have recently isolated a proteasome containing two different subunits only, the α-subunit with a molecular weight of 27 kDa and the β-subunit with a molecular weight of 25 kDa [5]. The amino acid sequence of the α-subunit derived from the nucleotide sequence, shows significant similarities to sequences of proteasome subunits from eukaryotes, including the presence of a putative tyrosine phosphorylation site and a motif with the features of a nuclear location signal. It is likely therefore that the proteasome gene family is of ancient origin [6].

Proteasomes from eukaryotes and from *Thermoplasma* have the same basic architecture: on electron micrographs they appear as cylinder-shaped complexes [5]. A recent three-dimensional analysis of the *Thermoplasma* proteasome by means of electron tomography has shown that the particle is more adequately described as a cylinder-shaped barrel, made of two 'outer disks' and two 'inner rings'. A tripartite inner compartment can clearly be recognized: the central compartment is bounded by the 'inner rings', and the two peripheral compartments are each delimited by one 'inner ring' and one 'outer disk' [7]. While the *Thermoplasma* proteasome must contain multiple copies of each of the two subunits (α, β) the larger number of different, though homologous subunits found in the eukaryotic proteasome implies that these will occur with small copy numbers in each particle.

In order to elucidate the molecular basis of the functions performed by proteasomes it is necessary to determine the arrangement of the subunits in the complex. In this communication we report on the distribution of the α- and β-subunits in the *Thermoplasma* proteasome, as determined by electron microscopy, using antibodies specific for the two subunits.

Abbreviations: IgG, immunoglobulin G; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid.

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2. MATERIALS AND METHODS

2.1. *Preparation of antisera, isolation and purification of immunoglobulin G*

The isolation and purification of proteasomes from *Thermoplasma acidophilum* and the electrophoretic separation of the subunits were carried out as described previously [5]. Antisera against the purified α- and β-subunits as well as the whole proteasome were raised in rabbits by intradermal injections of 6, 10 and 20 μg of each protein, respectively. The antigens were dissolved in a solution of 25% (v/v) Freund's complete adjuvans, 20 mM Tris-HCl, 1 mM EDTA, 1 mM NaN_3, pH 7.5. After 4 weeks an intravenous booster injection of 5 μg of each protein dissolved in 20 mM Tris-HCl buffer, pH 7.2, was given. IgG fractions of the antisera were isolated by chromatography on

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Protein *A* Superose (Pharmacia/LKB) using the following procedure: 1 ml of rabbit serum was mixed with 1 ml 100 mM Tris-HCl, pH 7.2 and then filtrated through a Millex GV filter (Millipore). The diluted antiserum was then subjected to a Protein *A* Superose (or Protein A Sepharose 4B-CL) column equilibrated with the same Tris-buffer, which was also used to wash out unbound material of the column. Bound IgG was eluted from the resin either with 100 mM glycin-HCl, pH 3.0, which was neutralized with 1 M Tris solution, immediately or with 3 M KSCN dissolved in phosphate-buffered saline (PBS) and the eluate exhaustively dialyzed against PBS. The IgG solution was concentrated by ultrafiltration and in some cases gel-filtrated on a column of superose 6 prep grade (Pharmacia/LKB) equilibrated with 100 mM Tris-HCl, pH 7.2.

As the antiserum raised against the β-subunit was slightly contaminated with antibodies reacting with the α-subunit, these antibodies were further purified by immunoadsorption on the β-subunit, which was separated from the α-subunit by SDS-PAGE and immobilized by Western blotting on Immobilon P membrane (Millipore) [5]. The antibodies adsorbed were eluted from the membrane with 0.2 M glycin, pH 2.8, neutralized with 1 M NaOH and then dialyzed against 100 mM Tris-HCl, pH 7.2.

For some experiments the anti-α-subunit antisera were affinity-purified on a CNBr-activated sepharose 4B-CL column (Pharmacia/LKB, Uppsala, Sweden) to which the whole enzyme had been coupled (1 mg/ml swollen gel). In order to maintain the structure of the whole complex during the harsh antibody elution conditions, the Sepharose-coupled enzyme had been crosslinked with 0.1% glutaraldehyde in PBS, pH 7.5, for 1 h. 1 ml of the antiserum was diluted 1:10 with PBS, pH 7.5 and circulated for 3 h (flow-rate 15 ml/h) over the column, which had been equilibrated with PBS. After unbound material had been washed out with PBS, the antibodies were released from the column by eluting with 3 M KSCN as described above.

2.2. *Formation of proteasome-antibody complexes for immunoelectron microscopy*

Immunoelectron microscopy was carried out independently in the two laboratories (Max-Planck-Institut für Biochemie, Martinsried, and Diabetes Forschungsinstitut, Düsseldorf). Although various protocols were explored for antigen–antibody complex formation, essentially the following two procedures were applied:

Thermoplasma proteasomes and IgG at a molar ratio of 1:20 or 1:50 were mixed in a volume of 0.2 or 1 ml and incubated for 16–48 h at 4°C. The mixture (incubation volumes of 1 ml were concentrated in a Centricon-30 microconcentrator before) was subjected to gel filtration on Superose 6. We used Superose 6 prep grade equilibrated with 20 mM Tris-HCl, 1 mM EDTA, pH 7.5, as well as Superose 6 (HR 10/30) equilibrated with 100 mM Tris-HCl, 1 mM EDTA, 1 mM NaN_3, pH 7.5. The columns were run according to the instructions given by the manufacturer. The fractions containing the proteasome-antibody complexes preceded the two peaks of unbound proteasomes and antibodies. The former were pooled and concentrated (if necessary with a concomitant exchange of 100 mM to 10 mM Tris buffer) in Centricon-30 microconcentrators in order to obtain protein concentrations suitable for immunoelectron microscopy.

When antibodies against the α-subunit were used, which had been repurified by chromatography on Sepharose-coupled proteasomes, these antibodies were incubated with a 2- to 5-fold molar excess over *Thermoplasma* proteasomes (6 μg) in a volume of 50–120 μl for 16 h at 4°C.

2.3. *Electron microscopy and image processing*

Aliquots of proteasome–antibody complexes were applied to electron microscopy grids covered with a carbon film, which had been rendered hydrophilic by a 15 s exposure to glow discharge in a plasma cleaner (Harrick Scientific Corporation, New York). Samples were stained for 30 s with 1% (w/v) uranyl acetate, pH 4.1, or 2% (w/v) uranyl oxalate, pH 6.9. Electron micrographs were recorded with a Philips EM 420 at a magnification of 49 000 or 60 000 and an accelerating voltage of 80 kV or with a Siemens Elmiskop 102 (100 kV, magnification 50 000).

Images were screened visually for the presence of a suitable density of proteasome–antibody complexes. Areas of 256 × 256 pixels containing complexes with optimum contrast were digitized with an EIKONIX-microdensitometer and a step size of 15 μm, corresponding to 0.31 nm at the specimen level. Frames of 128 × 128 pixels containing single proteasome–antibody complexes were interactively extracted, centered by hand, classified by visual inspection and low-pass filtered.

2.4. *Other methods*

Electrophoresis in 15% polyacrylamide SDS-containing gels was performed according to the method of Laemmli [8]. Subsequently, the proteins were transferred onto Immobilin P membrane by semi-dry blotting. Blots were treated with antibodies and antigen–antibody complexes were visualized by using alkaline phosphatase conjugated anti-rabbit-IgG antibodies as described elsewhere [5].

3. RESULTS AND DISCUSSION

Information on the topography of subunits in a molecular complex obtained by antibody labelling critically depends on the specificity of the antibodies being used. Reaction of anti-α- and anti-β-subunit antibodies with Western blots of their respective antigens after their separation by SDS-PAGE revealed some cross-reaction of the anti-β-subunit antiserum with the α-subunit. This could either be due to contaminating anti-α-subunit antibodies or it could be due to the presence of epitopes common to both subunits. Therefore the anti-β-subunit antibodies were further purified by im-

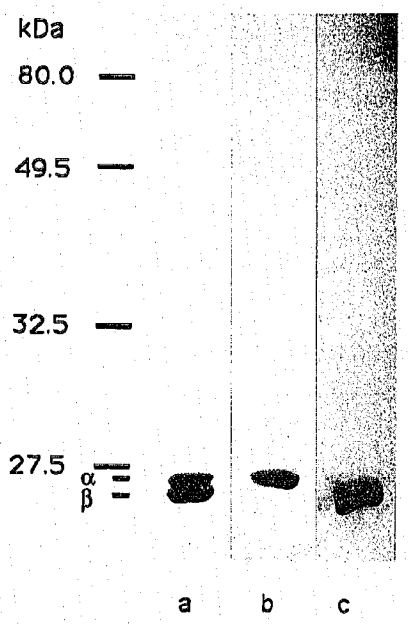

Fig. 1. 5.7 μg of purified *Thermoplasma* proteasomes were subjected to SDS-PAGE [6]. After electrophoresis (at 100 mA for 150 min without cooling) the proteins were transferred onto Immobilon P membrane by semi-dry blotting (10 V for 36 min at 6°C). Blocking and visualization of antigen–antibody complexes after reaction with (a) 1 μg/ml anti proteasome (whole particle) IgG, (b), 1 μg/ml anti-proteasome (α-subunit) IgG and (c) 1 μg/ml anti-proteasome (β-subunit) IgG were performed as described elsewhere [3].

munoadsorption to the β-subunit. The adsorbed antibodies were eluted by acidification as described in section 2. The specificity of the purified anti-β-subunit antibodies was tested in immunoblots (Fig. 1). There was no detectable immunological cross-reaction, indicating monospecificity of the antibodies for their respective antigens.

Interpretation of images of antibody labelled molecules requires some knowledge of the three-dimensional structure of the molecule. On electron micrographs of negatively stained preparations the cylinder or barrel-shaped proteasome complex shows two basic projections, rectangular side-views with a characteristic pattern of four striations and ring-shaped end-on views. Upon averaging, the end-on views show a weak but significant 7-fold symmetry [5,9].

At the chosen antigen-antibody ratio the majority of proteasomes interacts with the anti-α- and anti-β-subunit antibodies forming complexes dimeric with respect to the proteasomes (Fig. 2). With anti-α-subunit antibodies approximately 80% of all types of proteasome–antibody complexes observed are dimeric with the two combining sites of the IgG molecule attached to the two outer disks (Fig. 2a). Proteasomes joined together by a

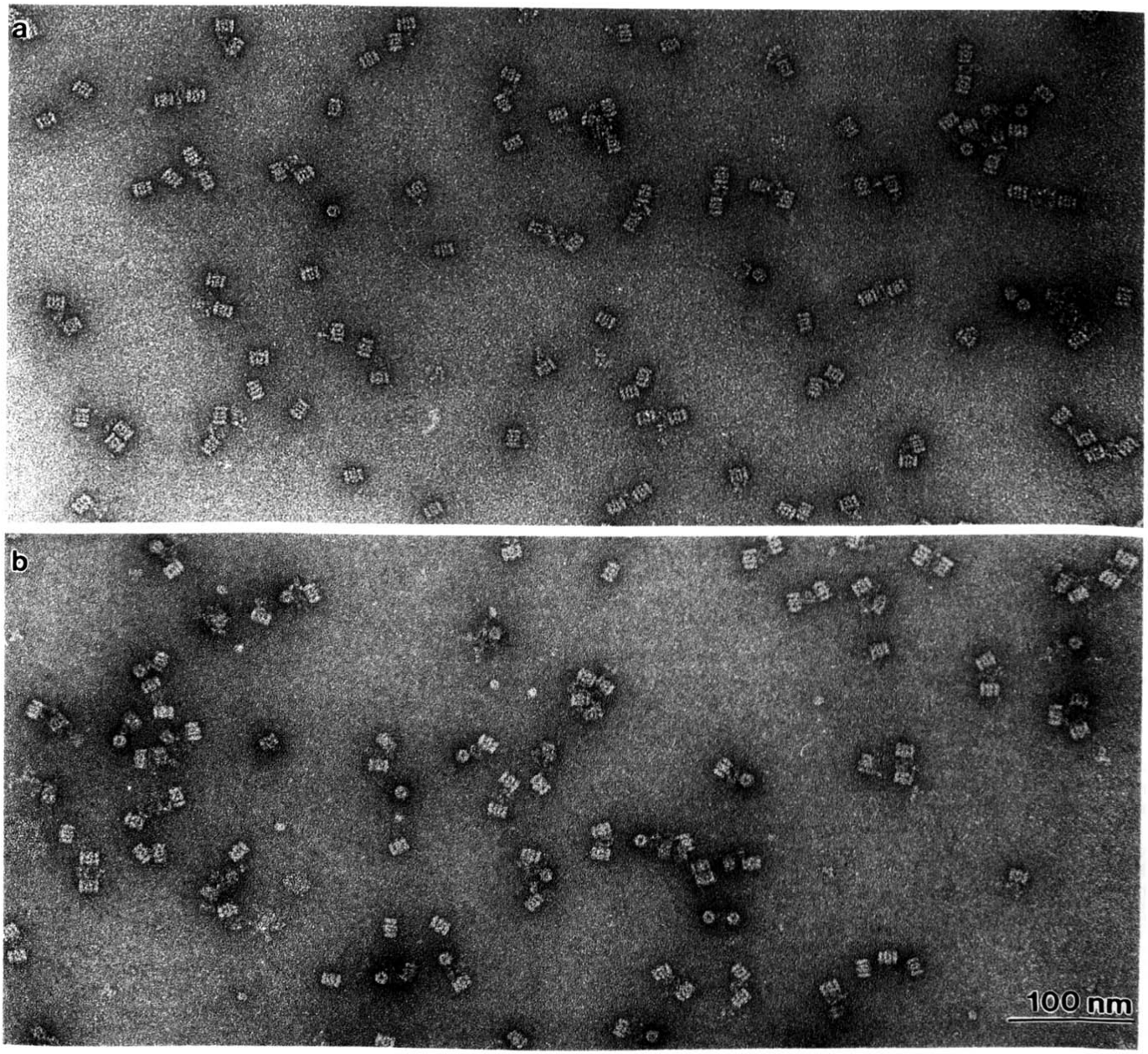

Fig. 2. Electron micrographs of negatively stained *Thermoplasma* proteasomes reacted with (a) anti-α-subunit IgG and (b) anti-β-subunit IgG. While proteasomes joined together by anti-α-subunit IgG show a variety of basic configurations (end-to-end, side-by-side or end-to-side) the majority of proteasomes cross-linked by anti-β-subunit IgG appear side-by-side.

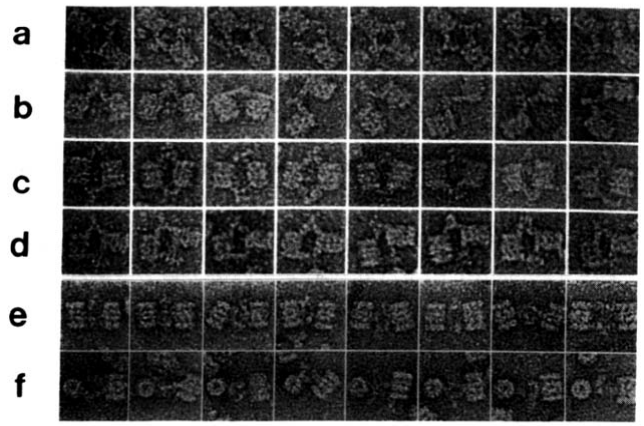

Fig. 3. Gallery of images of proteasome–antibody complexes showing the most abundant configurations (a–d) are proteasome-anti-α-subunit IgG complexes, (e,f) proteasome-anti-β-subunit IgG complexes: (a) shows the proteasomes cross-linked by two IgG molecules in the end-to-end configuration; (b) shows proteasomes joined together by a single IgG molecule; owing to the flexibility of the antibody the configuration varies from side-to-side to nearly end-to-end; (c) shows proteasomes cross-linked by two IgG molecules in the side-by-side configuration, and (d) proteasomes joined together by two IgG molecules in an end-to-side confuguration. In any case the anti-α-subunit IgG molecule is attached to the two disks at the opposite ends of the proteasome. In (e) the proteasomes are in the side-by-side configuration, most of them linked together by two anti-β-subunit immunoglobulins; in (f) the proteasomes are cross-linked such, that one of them is in the side-on and one in the end-on orientation. In any case, the anti-β-subunit IgG molecule is attached to the inner rings.

single IgG molecule have a higher degree of flexibility creating variable arrangements of the two proteasomes with respect to each other (Fig. 3b). Proteasomes joined by two anti-α-subunit antibodies are more restricted in their spatial arrangement and appear end-to-end, side-to-side or end-to-side (Fig. 3a,c,d). Especially in the end-to-end configurations (Fig. 3a) the antibody binding to the two outer disks is clearly recognizable (see also Fig. 4a).

With the anti-β-subunit antibodies approximately 90% of all the proteasome–antibody complexes show the proteasomes side-by-side with either one or two IgG

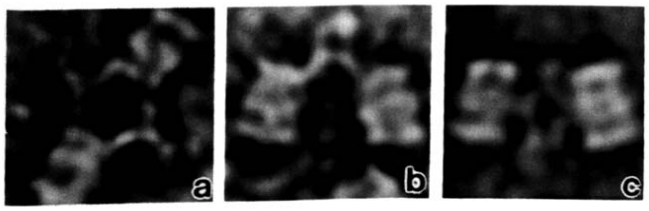

Fig. 4. Selected proteasome–antibody complexes magnified and low-pass filtered to remove high frequency noise: (a) shows two anti-α-subunit immunoglobulins attached to the outer disks of two proteasomes arresting them in the end-to-end configuration; (b) shows one anti-α-subunit IgG cross-linking two proteasomes; again the binding site can clearly be mapped to the outer disks; in (c) two proteasomes, side-by-side, are cross-linked by two anti-β-subunit immunoglobulins, attaching to the inner rings.

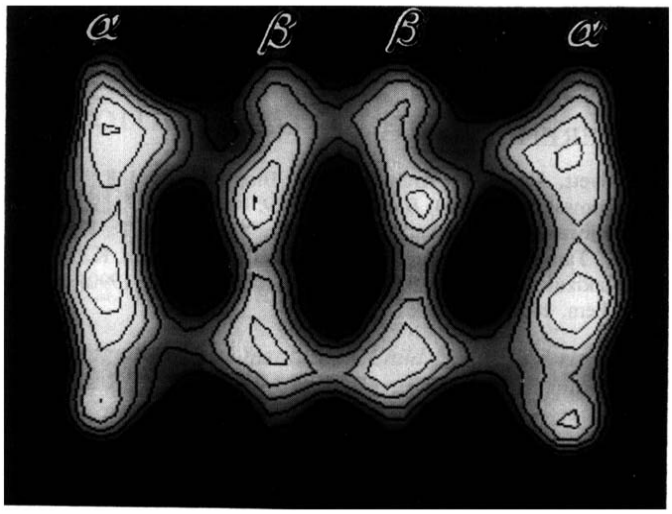

Fig. 5. An average of a *Thermoplasma* proteasome in the side-on orientation, summarizing the assignment of the α- and β-subunits.

molecules attached to the two juxtaposed inner rings. In many such complexes the cylinder axes are almost parallel to each other (Fig. 3e). More often than with anti-α-subunit antibodies one proteasome in the side-on-orientation and one in the end-on-orientation are joined together. Here too, the IgG appears to bind to the central part of the proteasome seen side-on (Fig. 3f). As compared to the manifold arrangements of anti-α-subunit antibody–proteasome complexes, fewer different configurations are encountered with the anti-β-subunit antibodies, obviously for steric reasons.

In an attempt to improve the visibility of the binding sites of the antibodies, selected IgG-proteasome complexes were magnified and low-pass filtered to remove the high frequency noise. The resulting images (Fig. 4) clearly show the binding of the anti-α-subunit antibody to the outer disks (Fig. 4a,b) and of the anti-β-subunit antibody to the inner rings (Fig. 4c).

In view of the highly conserved quaternary structure of proteasomes we expect that the eukaryotic homologues of the α- and β-subunit, respectively, occupy equivalent positions in the complex, i.e. that the homologues of the α-subunits form the disks closing off the 'barrel' at both ends, while the β-subunit homologues constitute the central part of the barrel (Fig. 5). It should be mentioned in this context that the α-subunit carries a sequence motif with the features of a nuclear location signal as well as a sequence complementary to it and several potential phosphorylation sites, suggesting that this subunit and its eukaryotic homologues serve a regulatory and/or targeting function. The catalytic (proteolytic) activity cannot yet be assigned to one of the two subunits.

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