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The 26S proteasome of the yeast *Saccharomyces cerevisiae*

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Abstract Proteasomes are large multicatalytic proteinase complexes found in all eukaryotic organisms investigated so far. They have been shown to play a central role in cytosolic and nuclear proteolysis. According to their sedimentation coefficients two types of these particles can be distinguished: 20S proteasomes and 26S proteasomes. In contrast to 20S proteasomes, which were mainly characterized on the basis of their ability to cleave small chromogenic peptide substrates and certain proteins in an ATP-independent manner, 26S proteasomes degrade ubiquitinylated proteins in an ATP-dependent reaction. 20S proteasomes have been found in all eukaryotes from yeast to man. So far 26S proteasomes have only been discovered in higher eukaryotes. We now report the existence of the 26S proteasome in a lower eukaryote, the yeast *Saccharomyces cerevisiae*. Formation of the 26S proteasome could most effectively be induced in crude extracts of heat stressed yeast cells by incubation with ATP and Mg^{2+} ions. This treatment yielded a protein complex, which eluted from gel filtration columns at molecular masses higher than 1500 kDa. Besides chromogenic peptide substrates, this complex cleaves ubiquitinylated proteins in an ATP-dependent fashion. In non-denaturing-PAGE, the purified 26S proteasome disintegrated and migrated as four protein bands. One of these bands could be identified as the 20S proteasome. On SDS-PAGE, the 26S proteasome showed a complex pattern of subunit bands with molecular masses between 15 and 100 kDa. Further evidence for the 20S proteasome being the proteolytically active core of the 26S proteasome was obtained by following peptide cleaving activities in extracts of yeast strains carrying mutations in various subunits of the 20S proteasome.

Key words: Proteolysis; Ubiquitin; 26S Proteasome; Yeast; *Saccharomyces cerevisiae*

1. Introduction

Proteasomes are large proteinase complexes which fulfil central functions in the eukaryotic cell [1–4]. Genetic evidence shows, that these functions range from the general feature of degrading ubiquitinylated proteins [5–9] to the specific action in degrading non-assembled protein subunits [10], regulatory proteins [11,12] and metabolically regulated enzymes [13–15]. Two types of proteasomes are known, which according to their sedimentation coefficients are called 20S and 26S proteasomes.

20S proteasomes (molecular mass about 700 kDa) are found in eukaryotic cells from yeast [16] to man [17]. They are cylindrically shaped particles composed of a multitude of different subunits with molecular masses between 22 and 32 kDa arranged in a stack of four, most probably seven-membered, rings [1,18]. A structurally similar but simpler anchestral proteasome has been discovered in the archaebacterium *Thermoplasma acidophilum* [19]. It is made up of only two types of subunits: α subunits, that are positioned at the outer rings and β subunits that are located at the juxtaposed inner rings of the cylindrical particle [20]. In contrast, a whole family of different, but related proteins is used to make up the more complex eukaryotic 20S proteasome. 55 members of this 20S proteasome gene family have been cloned so far from different sources. Sequence comparison divides them into two groups, one related to the α-, the other related to the β-subunit of the *Thermoplasma* proteasome [21]. Each group further splits into 7 subgroups [22]. 14 subunits have been cloned and sequenced from the yeast *Saccharomyces cerevisiae*, and each subunit can be allocated to one of the 7α and 7β subgroups [22]. From these findings, as well as from electron microscopic imaging [18,23] the eukaryotic proteasome has been proposed to represent a complex dimer composed of two identical subcomplexes with $\alpha_7\beta_7$ stoichiometry. In this structure, each of the 14 subunits is present in two copies located at defined positions.

Eukaryotic 20S proteasomes exhibit different peptide cleaving activities, which are able to split synthetic chromogenic peptide substrates at the carboxyterminal side of hydrophobic, acidic or basic amino acids. These activities have been referred to as chymotrypsin like-, peptidyl-glutamyl-peptide-hydrolyzing (PGPH)- and trypsin-like activity, respectively [1–4]. Recently, additional activities cleaving at branched chain or small neutral amino acids have been detected [24]. 20S proteasomes are also able to degrade certain proteins in vitro in an ATP-independent fashion [1, 25–29].

A 26S proteasome has been discovered in lysates from rabbit reticulocytes [30, 31]. This higher molecular mass species could be shown to exist in a number of tissues of higher eukaryotes [32–36]. In contrast to the 20S complex, the 26S proteasome degrades ubiquitinylated proteins in an ATP-dependent fashion in vitro [31] (for reviews see [4,37,38]).

The 26S proteasome (molecular mass of about 1500–2000 kDa [30, 31]) is assembled in vitro in an ATP-dependent manner from the 20S proteasome as catalytic core particle and at least two additional factors termed CF-1 and CF-2 [30,39,40]. CF-1 and CF-2 seem to make up the '19S-Cap' subcomplex, which can be detected in electron microscopic images to be attached to both ends of the central 20S proteasome 'barrel'

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Abbreviations: AMC, amidomethylcoumarine; βNA, β naphthylamide; BSA, bovine serum albumin; Cbz, benzyloxycarbonyl; DMSO, dimethylsulfoxide; DTT, dithiothreitol; MoβNA, 4-methoxy-β naphthylamide; oxidized RNAse A, oxidized ribonuclease A; PGPH, peptidylglutamyl-peptide-hydrolyzing; Suc, succinyl; TCA, trichloroacetic acid; Ub-, ubiquitinylated.

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[41,42]. A number of 26S proteasome subunits [43–47] turned out to be members of a new ATPase family [48–50].

The discovery of a 20S proteasome in the yeast *S. cerevisiae* (originally termed proteinase yscE [16,51]) enabled extensive biochemical studies which provided the basis for a profound genetic and molecular biological characterization of this particle [5–7,22,52–54]. However, the existence of a particle homologous to the 26S proteasome of higher eukaryotes in *Saccharomyces cerevisiae* could not be shown so far. Taking into account the high homology of the yeast 20S proteasome with the mammalian 20S complex, the existence of the 26S proteasome also in yeast seemed most probable. Genetic evidence has already been accumulating during the past years, which made the existence of a 26S particle in yeast very likely. Mutants harbouring defects in subunits of the yeast 20S proteasome accumulate ubiquitinylated proteins [5–7] and stabilize substrates of ubiquitin dependent proteolysis in vivo [8,9,12]. Ubiquitinylated proteins can only be degraded by the 26S proteasome in vitro. Additionally several genes have been cloned recently in *Saccharomyces cerevisiae*, the products of which were found to be highly homologous to previously discovered subunits of the 26S proteasome in higher eukaryotes [46,47].

Here, we show, that the 26S proteasome exists in yeast. The formation of this high molecular mass complex can be induced in crude extracts from yeast cells grown in selective mineral (MV) medium under heat stress by incubation with Mg^{2+} ions and ATP.

2. Materials and methods

2.1. Equipment and chemicals

Fluorogenic peptide substrates were obtained from BACHEM (Basel, Switzerland). FPLC equipment, Sepharose CL-4B, Sephacryl S400, Superose 6 prep. grade and protein standards for column calibration were supplied by Pharmacia (Freiburg, Germany). Creatine kinase, creatine phosphate and yeast hexokinase were from Boehringer Mannheim (Mannheim, Germany). ATP was purchased from FLUKA (Buchs, Switzerland) and 2-deoxyglucose from Merck (Darmstadt, Germany). French Press was from SLM Instruments (Urbana, Ill, USA) and ultrafiltration equipment was from AMICON (Beverly, MA, USA).

2.2. Yeast strains

Saccharomyces cerevisiae strains ABYS 1 (*MATa/MATα pra1-1/pra1-1 prb1-1/prb1-1 prc1-1/prc1-1 cps1-3/cps1-3 ade/ade*), YMF2 (*MATα* pra1-1 prb1-1 prc1-1 cps1-3 ura3*Δ5 his⁻ leu2-3,112 pre 2-1*) or YMF4 (*MATα pra1-1 prb1-1 prc1-1 cps1-3 ura3Δ5 his⁻ leu2-3,112 pre4-1*) were used as indicated.

2.3. Cell cultures and growth media

Precultures were grown for 48 h at 30°C in YPD medium (1% yeast extract, 2% peptone and 2% glucose). Main cultures were inoculated with 1/666 of the preculture and cells were grown in selective mineral (MV) medium (0.67% yeast nitrogen base without amino acids, 2% glucose, containing 0.3 mM adenine, 0.2 mM uracil. 1.66 mM leucine and 0.03 mM histidine were additionally added, when strains YMF3 and YMF4 were cultivated). Cells were grown until cell density exceeded 10^7 cells/ml and glucose content had dropped below 0.5% (15 h at 30°C and 25–27 h at 38°C).

2.4. Preparation of crude extracts

Cells were harvested by centrifugation at 2,000 × *g* for 10 min (yield about 4 g wet weight/l culture) and resuspended in buffer (30 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2 mM DTT, 5 mM ATP and 5 mM $MgCl_2$). Crude extracts were prepared by passing the cell suspension four times through a french pressure cell at 4°C with 1.1×10^8 Pa (1,100 bar, 16,000 psi). Cell breakage was controlled microscopically. Immediately after breakage, glycerol was added to the cell extract to a final concentration of 15%. Centrifugation for 30 min at 4°C and 10,000 × *g* yielded a crude extract with a protein concentration of about 50 mg/ml.

2.5. Activity assays with fluorogenic peptide substrates

(i) Trypsin-like activity: 150 μl of enzyme aliquot and 40 μl 0.5 M Tris-HCl, pH 10, were mixed. 10 μl of 10 mM Cbz-Ala-Arg-Arg-Moβ NA solution in DMSO were added, and the test solution was incubated for 200 min at 37°C. After stopping the reaction by the addition of 200 ml 0.2 M citrate pH 6.4, 4% Tween 20, fluorescence emission was measured after excitation at 366 nm using a filter of 380–3,000 nm. One activity unit (U) is defined as μmol of fluorophore generated per minute under the test conditions.

(ii) PGPH (peptidylglutamyl-peptide-hydrolyzing) activity: 150 μl of enzyme aliquot and 40 μl 0.5 M Tris-HCl, pH 8, were mixed. 10 μl of 10 mM Cbz-Leu-Leu-Glu-βNA solution in DMSO were added, and the test solution was incubated for 200 min at 37°C. After stopping the reaction by the addition of 200 μl 0.2 M citrate, pH 6.4, 4% Tween-20 fluorescence emission was measured after excitation at 366 nm using a filter of 380–3000 nm. One activity unit (U) is defined as μmol of fluorophore generated per minute under the test conditions.

(iii) Chymotrypsin-like activity: 50 μl of enzyme aliquot and 100 μl 75 mM Tris-HCl pH 7.8, 3 mM ATP, 15 mM $MgCl_2$ were mixed. 10 μl of 1.5 mM Suc-Leu-Leu-Val-Tyr-AMC solution in DMSO were added, and the test solution was incubated for 30 min at 37°C. After stopping the reaction by the addition of 1 ml ice cold ethanol fluorescence emission was measured after excitation at 366 nm using a filter of 420–3,000 nm. One activity unit (U) is defined as μmol of fluorophore generated per minute under the test conditions.

2.6. Determination of protein concentrations

Protein concentration was determined according to the method of Bradford [55] using BSA as standard.

2.7. Gel chromatograpic separations

Column packing and calibration were performed according to the manufacturer's instructions (Pharmacia). Thyroglobulin (669 kDa), ferritin (440 kDa) and catalase (232 kDa) were used as molecular mass standards. Chromatographic separations were performed at 4°C.

2.8. Pre-incubation of crude extracts

Crude extracts were divided into two portions. To one portion (designated 'plus') an ATP-regenerating system consisting of 0.5 mM ATP, 10 mM creatine phosphate and 100 μg/ml creatine kinase was added. The other portion (designated 'minus') was depleted from ATP by addition of 20 mM 2-deoxyglucose and 25 μg/ml hexokinase. Both portions were incubated at 37°C for 30 min.

2.9. Gel filtration chromatography

Immediately after incubation, the 'minus' and 'plus' portions of the extract were applied onto identical Sepharose CL-4B columns (1.6 × 50 cm) equilibrated with degassed buffer A (30 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2 mM DTT and 15% (v/v) glycerol) and buffer B (30 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2 mM DTT, 15% (v/v) glycerol, 5 mM ATP and 5 mM $MgCl_2$), respectively. Elution was performed in parallel at 4°C at a flow rate of 0.25 ml/min and 4 ml fractions were collected.

2.10. Purification procedure of the Saccharomyces cerevisiae 20S proteasome

Purification of the yeast 20S proteasome was performed essentially as described [16] and modified according to [56].

2.11. Purification procedure of the Saccharomyces cerevisiae 26S proteasome

Chromatographies were performed at 4°C. Crude extracts were prepared and preincubated with the ATP-regenerating system as described above. Immediately after incubation, the extract was applied onto a Sepharose CL-4B column (3 × 120 cm) equilibrated with buffer B. Elution was performed at a flow rate of 0.5 ml/h and 6 ml fractions were collected. Chymotrypsin like activity was measured in all fractions. The proteasome activity fractions in the molecular mass range between 1,200 kDa and 2,000 kDa were pooled and concentrated approximately ten-fold by ultrafiltration using an AMICON YM30 membrane. The concentrate was applied onto a Sephacryl S400 column (1 × 60 cm)

equilibrated with buffer B. Elution was performed at a flow rate of 0.3 ml/h and 1.6 ml fractions were collected. Chymotrypsin like activity was measured in all fractions. Peak fractions were pooled and concentrated approximately five-fold by ultrafiltration using an AMICON YM30 membrane. The concentrate was applied onto a Superose 6 column (1 × 30 cm) equilibrated with buffer B. Elution was performed at a flow rate of 0.5 ml/h and 0.8 ml fractions were collected.

2.12. Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was performed as described by Laemmli [57] using 12% gels. In non-denaturing PAGE SDS was omitted. 6% gels were used and gels were run overnight (20 h) at 0°C for 1,000 Vh.

2.13. Silver staining of gels

Immediately after electrophoresis, gels were sequentially incubated in 10% acetic acid, 40% methanol (v/v) (5 min), water (5 min), 12.5% glutardialdehyde (7.5 min), water (two times 5 min), 20% ethanol (7.5 min), staining solution (1 ml 20% silver nitrate (w/v), 1 ml 25% ammonium hydroxide, 5 ml 4% NaOH (w/v) and 93 ml 20% ethanol yielding 100 ml) (7.5 min in the dark), 20% ethanol (two times 5 min), developer (100 μl 37% (w/v) formaldehyde and 25 μl 2.3 M citric acid in 100 ml 20% ethanol) (3 to 6 min) and finally in 5% glycerol, 10% acetic acid (v/v) (5 min).

2.14. Formation of ubiquitin-protein conjugates

Ubiquitin conjugates of histone ^{125}I-labeled H2A were prepared as described [58]. The reaction mixture contained in a final volume of 600 μl: 50 mM Tris-HCl, pH 7.6, 2 mM DTT, 5 mM $MgCl_2$, 2 mM ATP, 120 μg ubiquitin, 5 mM Arg-Ala, ^{125}I-histone H2A (20 μg protein; 60–90 × 10^6 cpm), 96 μU ubiquitin-activating enzyme, E1, 14 μU ubiquitin-conjugating enzyme, E2, and 37 μU ubiquitin-protein ligase, E3α (1 unit of enzyme catalyzes the incorporation of ubiquitin into conjugates at a rate of 1 μmol/min as described in [58,59]). Following incubation at 37°C for 30 min, formic acid was added to a final concentration of 20% and the reaction was incubated for additional 20 min at 37°C. Conjugates were resolved from unreacted labeled histone H2A by gel filtration chromatography over 1 × 50 cm column of Sephadex G-150-120 equilibrated with 5% formic acid, 0.2 mg/ml BSA and 0.02% sodium azide. Fractions of 1 ml were collected. Following determination of the radioactive profile of the column, fractions containing the radioactive conjugates were pooled and dialyzed against 50 mM Tris-HCl, pH 7.2, and 0.02% sodium azide followed by extensive dialysis against H_2O containing sodium azide. Labeled conjugates were stored at 4°C up to 6 weeks. ^{125}I-labeled conjugates of oxidized RNAse A were prepared in a similar manner, except that Arg-Ala was omitted and the conjugates were resolved on Sephadex G-100-120. Products of the reaction were analyzed by SDS-PAGE and were essentially free of unconjugated labeled proteins.

2.15. Degradation of labeled ubiquitin-protein conjugates

Synthesized conjugates were labeled in their core-protein moiety, so that degradation of this moiety could be followed. Reaction mixture contained in a final volume of 25 μl: 40 mM Tris-HCl, pH 7.6, 2 mM DTT, 5 μM $MgCl_2$, 2 mM ATP, ^{125}I-labeled protein-ubiquitin conjugates (1–2 × 10^4 cpm), 5 μl of 5-fold concentrated sample from the respective fractions, and in the case of ubiquitinated H2A, 5 μg of

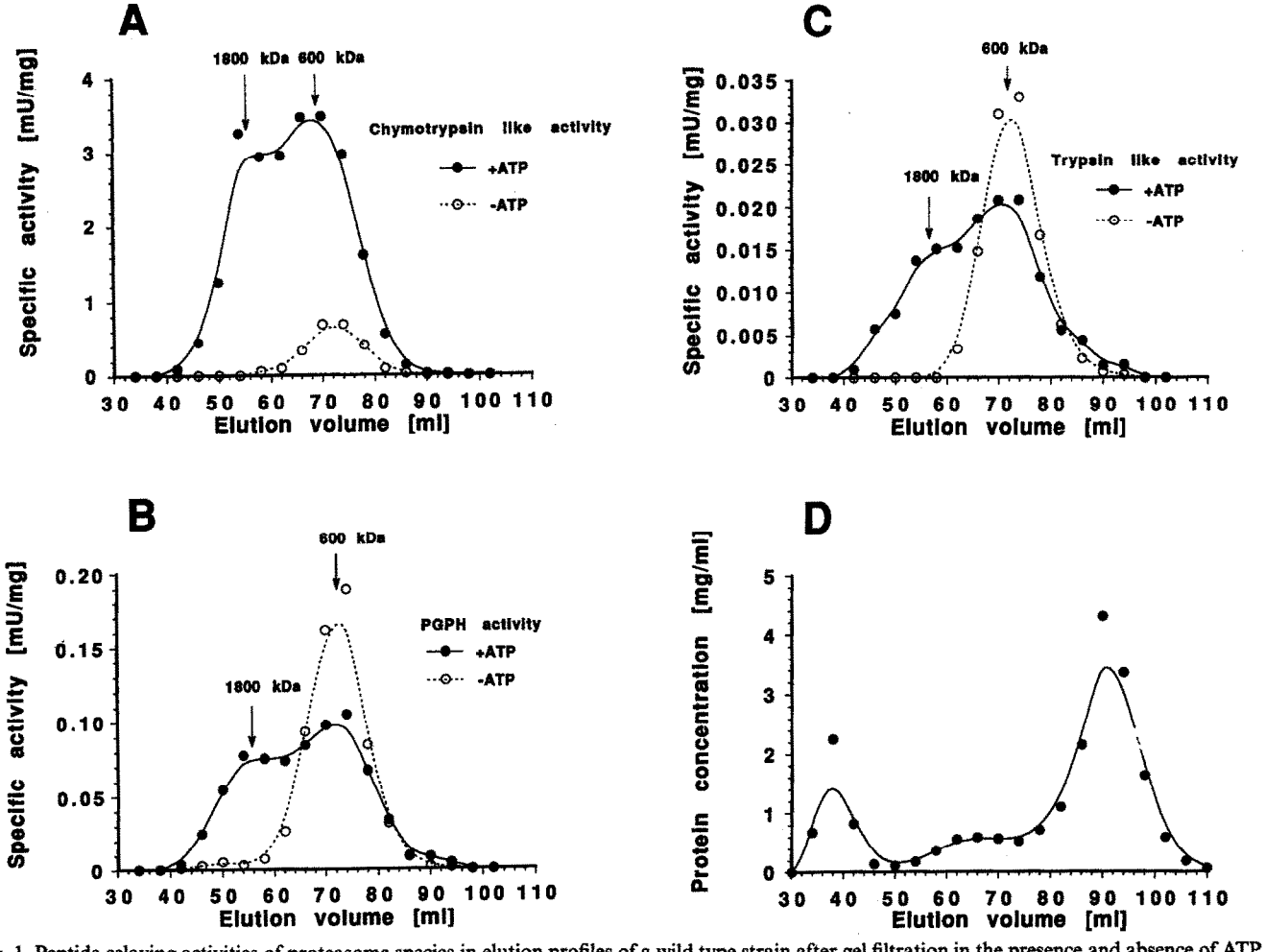

Fig. 1. Peptide celaving activities of proteasome species in elution profiles of a wild type strain after gel filtration in the presence and absence of ATP. Chymotrypsin-like (A), PGPH (B), trypsin-like (C) activities and the corresponding protein profile (D) are shown. Crude extract of *S. cerevisiae* strain ABYS1 was chromatographed after preincubation with ATP in ATP-containing buffer B (●) or after ATP depletion in ATP-free buffer A (○) in parallel on identical Sepharose CL-4B columns (1.6 × 50 cm). Molecular masses corresponding to the elution volumes are indicated at the peaks.

Sephadex G-100 purified FH/EF-1α [58,60]. The reaction was incubated at 37°C for 2 h, and a carrier protein was added (50 μl of a 1:1 mixture of 100 mg/ml BSA and crude human erythrocyte Fraction I). TCA was added to a final volume of 1 ml and a final concentration of 20%, and the tubes were left on ice for 15 min. Following centrifugation, radioactivity in the supernatant was determined in a τ-counter. Results are expressed as the fraction (%) of the labeled protein solubilized (100% is the amount of radioactivity in the TCA protein pellet from a reaction mixture that was incubated without the protease complex and FH/EF-1α).

3. Results

3.1. A 26S proteasome can be detected in yeast cells grown at elevated temperature

In order to detect the presence of a 26S proteasome in yeast, cells of *S. cerevisiae* strain ABYS1 were grown in mineral medium (MV) supplemented with adenine and uracil. Crude extracts were prepared as described in the methods section. One portion of the extract, designated 'plus', was incubated at 37°C with ATP and an ATP regenerating system consisting of creatin phosphate and creatine kinase, while a second portion, designated 'minus', was depleted from ATP with 2-deoxyglucose and hexokinase at 37°C. Immediataly after incubation for 30 min, 'plus' and 'minus' portions were subjected, in parallel, to gel chromatographic separation on identical Sepharose CL-4B columns. Elution buffer for the 'plus' run contained Mg^{2+} ions and ATP, whereas elution buffer for the 'minus' run was free of Mg^{2+} ions and ATP. Fractions were collected and trypsin like-, chymotrypsin like- and PGPH-activity as well as protein content were determined in each fraction. At standard temperatures of growth (30°C), only trace amounts of a 26S proteasome could be observed in cell extracts. However, when cells were grown under heat stress (38°C), a different picture emerged. Whereas in the 'minus' (ATP free) run the proteasomal peptide cleaving activities appeared in a single symmetrical peak at an elution volume corresponding to about

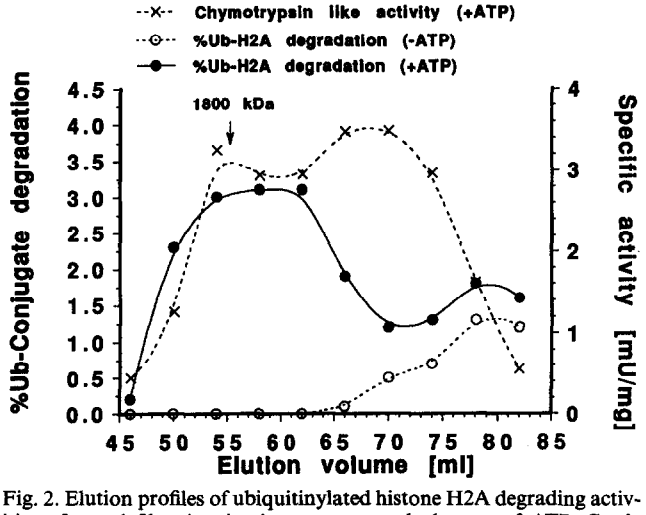

Fig. 2. Elution profiles of ubiquitinylated histone H2A degrading activities after gel filtration in the presence and absence of ATP. Crude extract of *S. cerevisiae* strain ABYS1 was chromatographed after preincubation with ATP in ATP-containing buffer B (●) or after ATP depletion in ATP free buffer A (○) in parallel on identical Sepharose CL-4B columns (1.6 × 50 cm). Molecular masses corresponding to the elution volumes are indicated at the peaks. Chymotrypsin-like activity in the presence of ATP is shown for comparison (×).

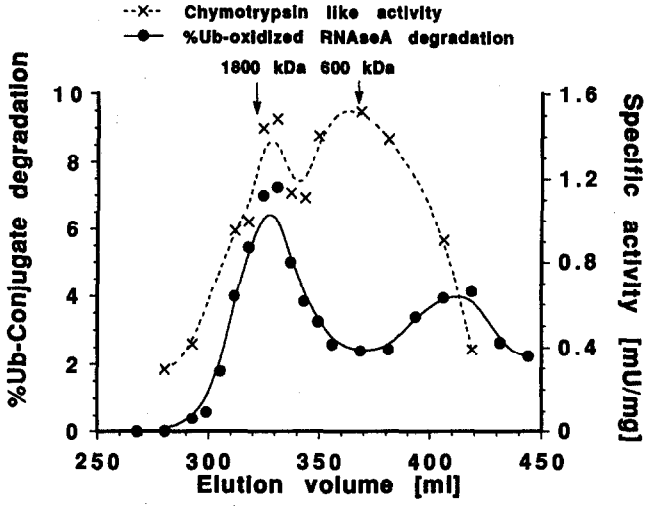

Fig. 3. Elution diagram of ubiquitinylated oxidized RNAseA degrading activity after gel filtration in the presence of ATP. Crude extract of *S. cerevisiae* strain ABYS1 was chromatographed on a Sepharose CL-4B column (3 × 120 cm) after preincubation with ATP in ATP containing buffer B. Molecular masses corresponding to the elutio volumes are indicated at the peaks. Chymotrypsin-like activity in the presence of ATP is shown for comparison (×).

600 kDa – the molecular mass of the 20S proteasome – in the 'plus' (ATP containing) run all three proteasomal activities showed a pronounced additional signal at an elution volume corresponding to a considerably higher molecular mass of about 1,800 kDa (Fig. 1A–D). Interestingly, when the chymotrypsin-like activity was measured (Fig. 1A) together with the appearance of the additional activity peak at 1,800 kDa, an increase of the activity at 600 kDa corresponding to the 20S proteasome can be observed.

3.2. The yeast 26S proteasome degrades ubiquitin protein conjugates

If the 'ATP-dependent' activity appearing at a molecular mass of 1,800 kDa represents the 26S proteasome, ubiquitin-protein conjugate degrading activity should be present in the corresponding fractions of the chromatography. Therefore, degradation of ubiquitin protein conjugates was measured in the fractions obtained from gel filtration chromatography. In the 'plus' (ATP containing) chromatography, activity degrading ubiquitinylated-histone H2A coelutes with the 1,800 kDa signal of peptide degrading activities (Fig. 2). No conjugate degrading activity is present in the fractions corresponding to the 1,800 kDa as well as in the 600 kDa molecular mass region of the 'minus' (ATP free) chromatography. A similar picture was obtained when ubiquitinylated oxidized RNAse A was used as a substrate (Fig. 3). A smaller signal of conjugate-degrading activity appeared in both ('plus' and 'minus') chromatographic separations at elution volumes corresponding to molecular masses below 600 kDa (see Figs. 2 and 3). We explain this behaviour as a reconstitution of 26S proteasome particles from their subcomponents induced by the action of ATP and Mg^{2+} in the activity assay.

3.3. Purification of the 26S proteasome

Initial attempts to purify the 26S proteasome of *S. cerevisiae* showed, that the particle is unable to tolerate most of the

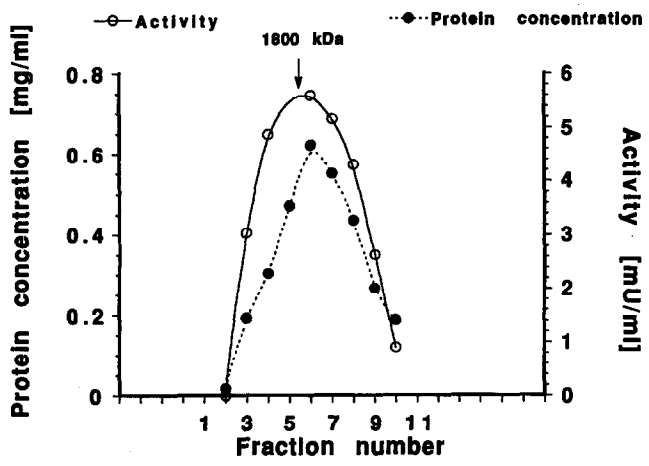

Fig. 4. Gel filtration chromatography of the 26S proteasome on a Superose 6 column (1 × 30 cm) in the presence of ATP. Buffer B was used for elution and the chymotrypsin-like activity is shown.

customary protein separation techniques. For instance, use of ion-exchange chromatography only yielded the 20S proteasome. To avoid interactions of the particle with the column matrix, gel filtration chromatographies were used. After three subsequent applications of this technique utilizing different column materials (Sepharose CL-4B, Sephacryl S400 and Superose 6), a single protein peak with a molecular mass of about 1,800 kDa was obtained (Fig. 4).

When the purified protein was subjected to non-denaturing PAGE in 6% gels, four bands could be distinguished (Fig. 5A, lane 2). The lowest of these comigrated with the purified yeast 20S proteasome (see Fig. 5A). After excision of this band from the non-denaturing gel and application to SDS-PAGE a protein pattern characteristic for the yeast 20S proteasome could be identified (see Fig. 5B). This clearly shows, that the 20S proteasome is part of the 1,800 kDa complex purified. We suggest, that the 26S proteasome disintegrates during non-denaturing electrophoresis yielding the 20S proteasome and other high molecular weight intermediates. The purified 26S complex was subjected to SDS-PAGE (12%) gels. A complex pattern of subunit bands in the range of 20 to 100 kDa appeared following silver staining (Fig. 6) as described for higher eukaryotic 26S proteasomes [32,36,39]. In addition, we could detect subunit bands with molecular masses below 20 kDa.

3.4. Studies on 20S proteasome mutants provide further evidence, that the 20S proteasome is a functional component of the 26S proteasome

To further demonstrate that the 26S proteasome contains the 20S proteasome as the proteolytically active core particle, we investigated the effect of point mutations in subunits of the 20S proteasome on the activity profiles obtained after gel chromatographies of extracts of respective mutant strains. For this experiment we used strain YMF4 carrying the *pre4-1* mutation, which completely lacks the PGPH activity of the yeast 20S proteasome, while the chymotrypsin like activity is unaffected by this mutation [7]. As can be seen in Fig. 7B, the presence of the *pre4-1* mutation completely abolishes the PGPH-activity in the 600 kDa and in the 1800 kDa region of the chromatographed extract. As expected, the chymotrypsin like activity shows wild type levels in both molecular mass regions (Fig. 7A). Additional experiments with strain YMF2 carrying the *pre2-1* mutation, which causes an almost complete loss of the chymotrypsin like activity leaving the PGPH activity unaffected [6], showed absence of the chymotrypsin like activity in the 600 kDa and in the 1,800 kDa molecular mass regions. The PGPH activity remained unchanged (data not shown).

4. Discussion

We have shown, that upon application of ATP and Mg^{2+} ions to yeast cell extracts, besides the well known 600 kDa molecular mass 20S proteasome, an additional peptide-cleaving activity with a considerably higher molecular mass appeared following gel filtration chromatography. This enzyme, that has a molecular mass in the region of 1,800 kDa is able to degrade ubiquitinylated proteins, a feature previously described for the higher eukaryotic 26S proteasome. This strongly suggests the existence of the 26S proteasome in the yeast *S. cerevisiae*. To our knowledge, this is the first time that the existence of this particle could be demonstrated in a microorganism. Electrophoretic analysis of the purified 26S complex and studies on mutants defective in peptide splitting activities of the 20S proteasome gave further support for the notion, that the 20S proteasome is a functional component of the 26S proteasome.

So far, the yeast 26S particle could be detected in considerable amounts only under certain conditions: (i) heat stress of 38°C had to be applied, (ii) cells had to be grown in mineral medium (MV) into late logarythmic phase and (iii) strains defective in the major vacuolar proteinases had to be used. All these conditions are known or believed to lead to considerably increased cytosolic proteolysis via the 26S proteasome [5]. Under heat stress, a higher amount of partially denatured and misfolded proteins is generated, which have been shown to be degraded by the proteasome system [5–7]. Recovery of amino acids by degradation of intracellular proteins may facilitate growth in mineral medium. In addition, in the late logarithmic growth phase, cells start adaptation to starvation conditions which requires reorganization of the metabolism and thus a higher rate of intracellular proteolysis [61]. Moreover, cells lacking the vacuolar proteinases may need increased cytosolic

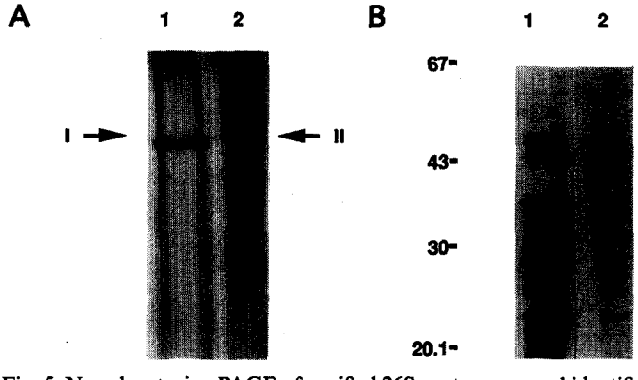

Fig. 5. Non-denaturing PAGE of purified 26S proteasome and identification of the 20S proteasome by SDS PAGE. (A) Lane 1, purified *S. cerevisiae* 20S proteasome for comparison. Lane 2, purified *S. cerevisiae* 26S proteasoem. (B) Bands indicated by arrows were excised from the non-denaturing gel in (A) and analyzed by SDS-PAGE. Lane 1, band I (purified 20S proteasome for comparison). Lane 2, band II. Gels were stained by silver. Molecular weight markers for SDS gels were BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20.1 kDa).

proteolytic degradation and therefore a higher amount of 26S proteasome to substitute for vacuolar proteolysis. Also, artifactual in vitro degradation of components of the 26S proteasome during the purification procedure may be avoided by the use of vacuolar proteinase deficient strains.

Purification of the 26S proteasome of *S. cerevisiae* by a series of gel chromatographic steps yielded an active protein which eluted as a single peak with a molecular mass of about 1,800 kDa. Non-denaturing PAGE led to the detection of four protein bands (Fig. 5A). The lowest band represents the 20S proteasome (Fig. 5B). We assign the uppermost of these bands to the intact 26S proteasome, whereas the faster migrating bands most likely represent intermediates generated by dissociation of the particle during electrophoresis. We interprete dissociation of the *S. cerevisiae* 26S proteasome even under the mild conditions of non-denaturing gel electrophoresis as a result of a greater lability of the yeast 26S complex as compared to the 26S particle in higher eukaryotes. This interpretation is also supported by our observation that harsher protein separation techniques, like ion exchange chromatography, could not be applied for purification of the yeast 26S proteasome.

On SDS-PAGE a rather complicated array of protein bands is visible when the purified 26S complex is analyzed. In the 20 kDa to 30 kDa range, a multitude of intense bands superimposing each other is visible, while several less intense bands appear scattered over the region between 30 kDa and 100 kDa. This subunit pattern is similar to the one described for the 26S proteasome in higher eukaryotic cells [32,36,39]. Few intense bands are visible below a molecular mass of 20 kDa (Fig. 6), which raises the possibility, that the subunit composition of the *S. cerevisiae* 26S proteasome differs from the composition in higher eukaryotes. However, these low molecular mass proteins may also represent contaminations inherent to the purification procedure.

The existence of the *Saccharomyces cerevisiae* 26S proteasome underlines the high evolutionary conservation of this proteolytic system among eukaryotes. Moreover, its discovery provides the basis for further investigation of the structure and function of this multifunctional proteinase complex. The unique applicability of genetic and molecular biological tools in the yeast *Saccharomyces cerevisiae* will provide further insight into the mechanism of cytosolic and nuclear proteolysis and the function of the 26S proteasome in the eukaryotic cell.

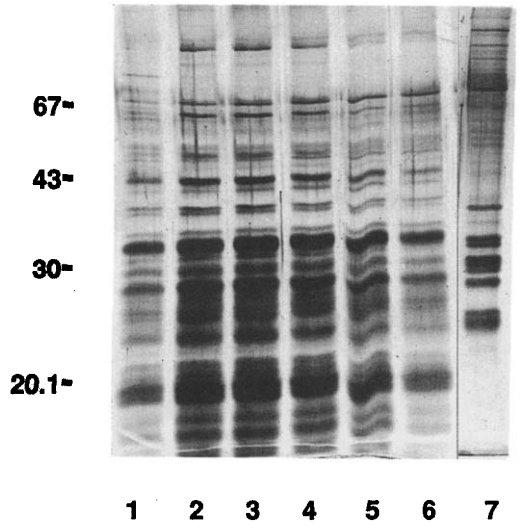

Fig. 6. SDS-PAGE of fractions containing the 26S proteasome following Superose 6 gel filtration. Lanes 1–6 correspond to fractions 3–8 of the final gel filtration step (Fig. 4). Lane 7, purified *S. cerevisiae* 20S proteasome for comparison. Gels were stained by silver. Molecular weight markers are as indicated in the legend to Fig. 5.

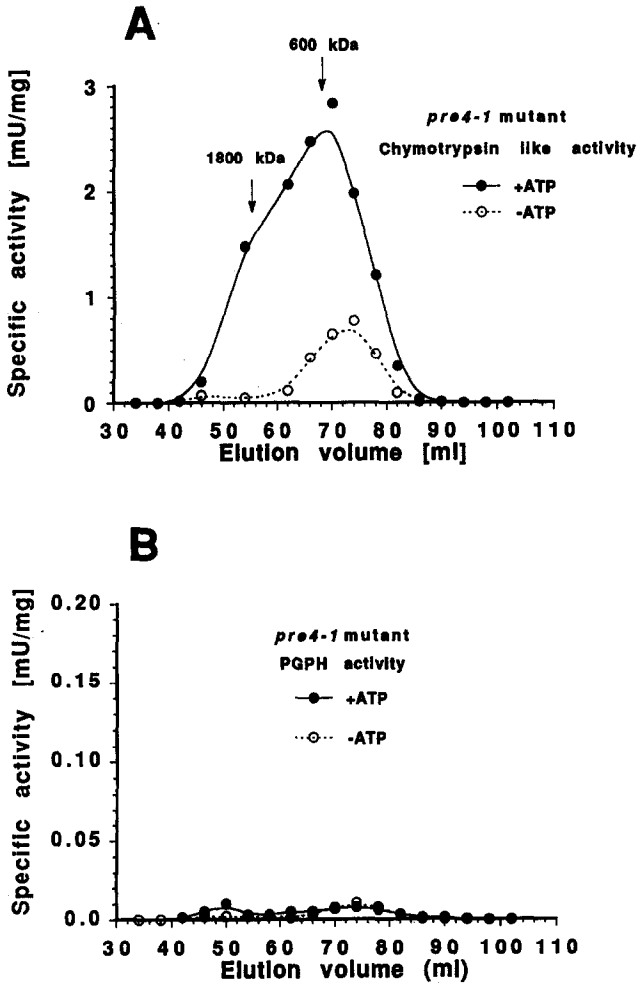

Fig. 7. Peptide-cleaving activities of proteasome species in elution profiles of a 20S proteasome mutant strain after gel filtration in the presence and absence of ATP. Chymotrypsin-like (A) and PGPH (B) activities are shown. Crude extract of strain YMF4 harbouring the *pre4-1* mutation was chromatographed after preincubation with ATP in ATP-containing buffer B (●) or after ATP depletion in ATP free buffer A (○) in parallel on identical Sepharose CL-4B columns (1.6 × 50 cm). Molecular masses corresponding to the elution volumes are indicated at the peaks.

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