The Regulatory Complex of *Drosophila melanogaster* 26S Proteasomes: Subunit Composition and Localization of a Deubiquitylating Enzyme

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Abstract. *Drosophila melanogaster* embryos are a source for homogeneous and stable 26S proteasomes suitable for structural studies. For biochemical characterization, purified 26S proteasomes were resolved by two-dimensional (2D) gel electrophoresis and subunits composing the regulatory complex (RC) were identified by amino acid sequencing and immunoblotting, before corresponding cDNAs were sequenced. 17 subunits from *Drosophila* RCs were found to have homologues in the yeast and human RCs. An additional subunit, p37A, not yet described in RCs of other organisms, is a member of the ubiquitin COOH-terminal hydrolase family (UCH). A analysis of EM images of 26S proteasomes-UCH-inhibitor complexes allowed for the first time to localize one of the RC’s specific functions, deubiquitylating activity.

The masses of 26S proteasomes with either one or two attached RCs were determined by scanning transmission EM (STEM), yielding a mass of 894 kD for a single RC. This value is in good agreement with the summed masses of the 18 identified RC subunits (932 kD), indicating that the number of subunits is complete.

Key words: protein degradation • ubiquitin • ubiquitin hydrolase • ATP-dependent proteolysis • electron microscopy

Introduction

In eukaryotic cells, the vast majority of cytosolic and nuclear proteins are degraded via the ubiquitin–proteasome pathway (Rock et al., 1994). Through a sequence of activating and ligating events, ubiquitin is covalently attached to proteins destined for degradation (for recent reviews see Varshavsky, 1997; Hershko and Ciechanover, 1998; Scheffner et al., 1998). Proteins carrying mult ubiquitin tags are selected by the 26S proteasome and degraded in an ATP-dependent process (Coux et al., 1996; Rechsteiner, 1998). The 26S proteasome is a large molecular machine built from ~30 different subunits that has an estimated molecular mass of 2,000–3,000 kD. Two major components jointly form the 26S (or more accurately, the 30.3S) complex: the barrel-shaped proteolytic core complex (the 20S proteasome) and the regulatory complexes (RCs), which associate with either one or both ends of the core complex (Peters et al., 1993; Yoshimura et al., 1993; see also Fig. 1).

Whereas the structure and enzymatic mechanism of the 20S proteasome have been studied in great detail (for recent reviews see Baumeister et al., 1998; Bochtler et al., 1999; Voges et al., 1999), current understanding of the structure and function of the RC is lagging behind. The RCs serve to recognize proteins carrying mult ubiquitin tags and to prepare them for degradation in the 20S proteolytic complex. The preparatory steps involve the binding of the ubiquitylated substrates, their deubiquitylation, the unfolding of the substrates, and finally, their translocation into the 20S complex (Lupas et al., 1993; Rubin and Finley, 1995). Substrate unfolding is required because admission to the active site chamber inside the 20S complex is restricted to unfolded polypeptide chains (Wenzel and Baumeister, 1995). A t the heart of the RCs is an array of

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**A abbreviations used in this paper:** 1D, one-dimensional; 2D, two-dimensional; 16-BAC, benzylcyclobutyl-n-hexadecylammonium chloride; AMC, 7-amido-4-methylcoumarin; MSA, multivariate statistical analysis;
A TPases, members of the A A A family (Confalonieri and Duguet, 1995; Beyer, 1997), which act as reverse chaperones (Braun et al., 1999; Strickland et al., 2000). RCs of
prokaryotic 20S proteasomes appear to have only a single type of A A A -ATPase (Wolff et al., 1998; Zwickl et al., 1999), which form homohexameric rings, whereas in eu-
karyotic RCs, six paralogs are found that are believed to
assemble into heterohexameric rings. The hallmark of all
proteasomal ATPases is an NH
z-terminal coiled-coil do-
main (Lupas et al., 1993; R echsteiner, 1998). In both pro-
teasomes and other self-compartmentalizing protein de-
gradation machines, the proteases and the ATPases form
colinear assemblies (Lupas et al., 1993; Zwickl et al.,
2000). Thus, the ATPases are well placed to unfold sub-
strates and control the gates that give access to the pro-
teolytic compartments (Larsen and Finley, 1997). Beyond
the ATPases, little is known about the roles of the other
~12 subunits of the RCs (for recent reviews see Tanaka
and Tsurumi, 1997; Voyges et al., 1999).

Structural studies with 20S proteasomes are hampered
by the low stability of the complexes, which tend to disso-
ciate into various subcomplexes. It has been shown previ-
sously that Drosophila melanogaster embryos provide a
rich source of 20S proteasomes (Udvardy, 1993) and yield
preparations that are sufficiently homogenous for struc-
tural studies (Walz et al., 1998). To prepare the grounds
for an in-depth structural analysis of the Drosophila RC,
we sought to establish a catalog of all its subunits; to assess
the completeness of this catalog, we have performed quan-
titative mass analysis using scanning transmission EM
(STEM). In the course of these studies, we identified a
novel subunit that turned out to be a deubiquitylating en-
zyme, p37A . Taking advantage of a nonhydrolyzable sub-
strate analogue, ubiquitin COOH-terminal aldehyde (Ub-
A), we have been able to map its location within the
complex providing new insights into the sequence of
events en route to substrate degradation.

Materials and Methods

Materials

Chromatography resins for protein purification were pur-
bred from Sigma-Aldrich, Amersham Pharmacia Biotech, Merck, Bio-
Rad, and Quiagen. Enzymes for DNA restriction and modification were
obtained from New England Biolabs, Inc. and Stratagene. Oligonucle-
otides for PCR reactions were synthesized on an Applied Biosystems
380A DNA synthesizer.

Isolation of 26S Proteasomes from Drosophila
melanogaster Embryos

26S proteasomes were purified as described previously (Udvardy, 1993;
Walz et al., 1998). In brief, 0–16-h Drosophila embryos (Y yellow white
strain) were collected at 25°C from feeding plates. A fter dechorination
and homogenization, the extract was clarified by centrifugation and nu-
cleic acids were removed by precipitation with 10% streptomycin sulfate.
The supernatant was fractionated with hydroxyapatite in a batch proce-
dure, followed by anion-exchange chromatography (diethylaminoethyl
cellulose, DE52, Whatman) and sucrose density gradient centrifugation
(15–40% sucrose). A t all stages, fractions were tested for their ability to
hydrolyze Succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Suc-
LLVY-AMC, Bachem), and only fractions containing the peaks of activity
were used for further purification. Protein concentrations were deter-
mained using the BioRad protein assay with BSA as standard.

Protein Gel Electrophoresis and Immunoblotting

SDS-PAGE was performed using 12.5% separating and 3.5% stacking
gels as described by Laemmli (1970). Protein samples were resolved by
nondenaturing PAGE using a modi-
fication of the method described by Hough et al. (1987). The resolving
gels were 4.6% acrylamide (37.5:1), 2.3% sucrose, 90 mM Tris, 80 mM bo-
rate, 0.08 mM EDT A , pH 8.3; polymerized with 0.04% ammonium persul-
phate and N,N,N,N'-tetramethyl-ethylenediamine. The non-denaturing
stacking gels contained the same buffer, but only 3.1% acrylamide (4:1). The
proteins were subjected to electrophoresis in 90 mM Tris, 80 mM bo-
rate, 0.08 mM EDT A , pH 8.3, for a total of 800 V-h (50 V for 16 h) at 4°C.
A fter electrophoresis, peptidease activity was detected by overlaying the
Gels with 100 μM Suc-LLVY-AMC in 5 mM M gCl2, 10 mM KCl, 0.5 mM
EDTA , 30 mM Tris, pH 7.8, for 1 h at 37°C. Protease bands were visu-
alized by exposure to UV light (360 nm) and photographs were taken be-
fore staining with Coomassie blue. A lternatively, proteins were trans-
ferred to nitrocellulose membranes by semidy blotting. Blots were
treated with antibodies and antigen-antibody complexes were visualized by
using alkaline phosphatase-conjugated anti-mouse IgG antibodies, fol-
lowing standard procedures (Sambrook et al., 1989).

For two-dimensional (2D) gels, 50 μg of purified 20S or 26S protea-
somes were concentrated by the use of N a n o s a l ™ microconcentrators
(Pall Filtron) and reuspended in 100 μl of loading buffer (9 M urea, 4% CHAPS, 40 mM Tris, and 0.025% Bromphenol blue). Separation in the
first dimension was performed with 13-cm Immobiline® Dry Strip Gels
(Amersham Pharmacia Biotech) using a linear pH gradient from 3–10.
A fter focusing for 66,500 V-h, the strips were positioned over a vertical
SD S-polyacrylamide slab gel made of a 5% stacking and 12.5% separating
gel, and subjected to electrophoresis using standard conditions. In addition, the benzylthio-dimethyl-hexadecylammonium chloride (16-BAC)/SDS-PA G in 2D gel electrophoresis system was used as described by Hartinger et al. (1996). Proteins were either stained in the gel with Coomasie blue or electrotransferred to polyvinylidene difluoride membranes for NH2-terminal sequencing.

**Protein Sequencing**

The protein-containing polyvinylidene difluoride membrane pieces were excised, cut into small pieces (3 × 3 mm), and incubated with 500 µl 0.2% polyvinylpyrrolidone (PVP 30) in water for 30 min at room temperature (Patterson, 1994). The supernatant was discarded and the membrane was washed six times with water and incubated with 0.1 M Tris-HCl, pH 8.0, 0.2 mM CaCl2, 10% acetonic, 1% nononylsphenoxy polyethoxylate ethanol (Tergitol NP-40), and 0.5 µg/ml endoproteinase LysC (Boehringer) for 8 h at 37°C. When the NH2-terminal was blocked, protein digestion was performed in the 2D gel. Therefore, gel pieces were excised, washed twice with cleavage-buffer (12.5 mM Tris, pH 8.5, 0.5 mM EDTA, i.e., half the usual concentration), and dried. Pieces of the gel were then incubated with endoproteinase LysC for 16 h at 37°C. The resulting cleavage fragments were eluted twice with 0.1% trifluoroacetyl and once with 10% formic acid, 20% isopropanol, and 20% acetonitrile. The supernatants were combined, dried, and the peptide mixture separated on a reversed phase column Purosphere RP-18 endcapped (Merck; 1 mm). The eluting solvent was water and 20 ml of 0.25% tannic acid, 0.2% sodium citrate, and 1 mM potassium carbonate were added to the vial. The peptides were sequenced (Edman and Begg, 1967) on a pulsed liquid phase sequencer, Procise 493 (Applied Biosystems). All sequences have been submitted to Genbank.

**DNA Sequencing**

The amino acid sequences obtained from peptide analysis were used to search the databases of the Berkeley Drosophila Genome Project (http://www.fruitfly.org) for matching DNA sequences. Five of the peptides led to genes described previously. All other peptides were homologous to distinct EST cDNA clones (Rubin et al., 2000, Table I). The longest cDNA clone of each EST clone was ordered from Genome Systems, Inc. or Research Genetics, and sequenced on both strands with a 373 DNA Sequencer. All sequences have been submitted to Genbank (EMBL/DBJ).

**Construction and Purification of Recombinant p37A**

The cDNA encoding the open reading frame for p37A was amplified by PCR, introducing an NH2-terminal NdeI and a COOH-terminal XhoI site. The amplified DNA fragment was subcloned into the prokaryotic expression vector, pET22b, fusing a (His)6-tag at the COOH terminus of the protein (Novagen Inc.). The construct was sequenced, confirming that no mutations had been introduced. Expression upon isopropyl-β-D-thiogalacto-pyranoside (IPTG) induction (1 mM, 5 h, 37°C) of E. coli BL21(DE3) cells (Studier et al., 1990) yielded a His-tagged protein of ~37 kD. The recombinant protein was purified on a nickel-nitrilotriacetic acid resin (Quiagen) and dialyzed against 50 mM Hepes, pH 7.8, 0.5 mM EDTA, 20% glycerol, 1 mM DTT.

**Determination of Ubiquitin COOH-terminal Hydroxylase Activity**

A assay for p37A and 265 proteasome enzymatic activity were performed essentially as described for the ubiquitin COOH-terminal hydroxylase (UCH-L1) enzyme (Dang et al., 1998). 5 µl 37A (~1 µM) and 20 µl 265 proteasomes (~0.5 mg/ml) were incubated in 490 and 475 (µl) of a 1 mM lactate-pyridoxal phosphate (Biorad) induction (11 μl, 5 h, 37°C) of E. coli BL21(DE3) cells (Studier et al., 1990) yielded a His-tagged protein of ~37 kD. The resulting cleavage fragment was incubated into a nickel-nitrilotriacetic acid resin (Quiagen) and dialyzed against 50 mM Hepes, pH 7.8, 0.5 mM EDTA, 20% glycerol, 1 mM DTT.

**Ubiquitinaldehyde–Colloidal Gold Conjugate**

Colloidal gold particles (3.5 nm), prepared by the method of Slot and Geuze (1989), were used with minor modifications. In brief, 80 ml of 0.205% sodium citrate, 20 ml of 0.25% sodium carbonate, and 1 mM potassium carbonate were heated to 60°C and rapidly mixed. The gold colloids were formed within seconds and no additional reagents were added. The gold colloids were mixed with 100 µl of 0.2% NaAuCl5 in water and 20 ml of 0.25% sodium carbonate, 0.2% sodium citrate, and 1 mM potassium carbonate were heated to 60°C and rapidly mixed. The gold colloids were formed within seconds and no additional reagents were added.

**Electron Microscopy**

2 µl of purified 265 proteasomes (0.2 mg/ml) was incubated with 1 µl of gold-labeled Ub-A1 and 2 µl of 20 mM Tris buffer, pH 7.2, for 7 min and applied to 100 x 400 mesh copper grids, which had been coated with carbon and glow-discharged in a plasma cleaner, for 45 s. After blotting and removing the sucrase with Tris buffer (20 mM), the preparation was nega-
tively stained with 2% aqueous uranyl acetate for 45 s. Electron micrographs were recorded digitally (Photometrics slow scan CCD; 1024 × 1024 pixels) at 45,700× using a CM 12 transmission electron microscope (Philips) at 120 kV accelerating voltage.

**Image Processing**

The images were transferred to a SGI workstation and analyzed using the EM software package (Hegerl, 1996). Subframes containing single 26S, either with or without gold particles, were interactively extracted from the images. The two sets of complexes were separately aligned, translationally and rotationally (5 cycles), using iterative cross-correlation techniques (Baumaster et al., 1988; Phipps et al., 1991). For the set where gold labels were present, the lower cutoff of the gray level range of each frame was increased to the mean value minus 0.5× SD before alignment to minimize the contribution of the gold particles. The alignment parameters were then assigned to the original stack. Subsequently, the dataset was subjected to multivariate statistical analysis (M SA).

**Scanning Transmission Electron Microscopy**

For mass analysis by STEM, the 26S proteasome stock preparation (150 µg/ml) was diluted 2–8 times in buffer without sucrose, and either used directly or after cross-linking with 0.05% glutaraldehyde (final concentration) for 5 min on ice. Aliquots of the solutions were immediately adsorbed to glow-discharged thin carbon films, supported by thick perforated carbon layers on gold-coated copper grids. In the absence of cross-linking, grids were washed 4–5 times with 0.1 M ammonium acetate. The glutaraldehyde-treated samples were washed several times with quartz bidi-stilled water. All grids were freeze-dried overnight in the microscope pretreatment chamber.

A Vacuum Generators STEM HB-5 interfaced to a modular computer system (Tietz Video; Image Processing Systems) and operated at 80 kV was used for the measurements. Series of 512 × 512-pixel digital images were recorded at a nominal magnification of 200,000×, using doses of ~300 electrons/Å². The data were evaluated using the IM PSYS program package as described previously (Müller et al., 1992). To this end, the particles were classified into eight groups according to their dimensions, the selection box was kept as small as possible. Total scattering within the selection box was determined and the scattering from an equivalent area of the background support film was subtracted to calculate the particle’s mass. The clearly identifiable top views of the strongly scattering 20S particles, present in all preparations, served as internal mass standards. The instrument’s calibration (Müller et al., 1992) allowed the use of a single scale factor for each experiment.

The mass of the 20S particle was taken as 721 kD, i.e., the average of the calculated mass values for the yeast and human 20S proteasomes. For each experiment (two glutaraldehyde and two ammonium acetate-treated preparations), mass data from the top views were displayed in a histogram and a Gaussian curve was fitted. The position of this peak compared with 721 kD yielded a global scale factor that was subsequently applied to the whole data set. In a final step, corresponding data sets from all four experiments were separately aligned, translationally and rotationally (5 cycles), using iterative cross-correlation techniques (Baumaster et al., 1988; Phipps et al., 1991). For the set where gold labels were present, the lower cutoff of the gray level range of each frame was increased to the mean value minus 0.5× SD before alignment to minimize the contribution of the gold particles. The alignment parameters were then assigned to the original stack. Subsequently, the dataset was subjected to multivariate statistical analysis (M SA).

**Results**

**Purification and Characterization of 26S Proteasomes from Drosophila Embryos**

The last step of proteasome purification, the sucrose density gradient centrifugation, yielded two peaks with high peptidase activity, capable of hydrolyzing the fluorogenic peptide, Suc-L-L-V-Y-A-M-C. The active fractions giving rise to each peak were analyzed by SD S-PA G E (Fig. 2) and nondenaturing PA G E (Fig. 3). Fraction 10, with ~22% sucrose, was the first fraction of peak 1 and contained pure 20S proteasomes. Fractions 13, 14, and 15 also contained 20S proteasomes, but these were contaminated with a high molecular weight protein that appeared similar to GroEL on electron micrographs. Fractions 16–20, from the second activity peak corresponding to ~26–28% sucrose, showed a pattern characteristic of 26S proteasomes on SD S gels. Under native conditions, the Drosophila complex separated into four bands, similar to the pattern observed with mammalian and yeast proteasomes (Hoffman et al., 1992; Glickman et al., 1998a). Fluorogenic peptide overlays showed, besides 20S proteasomes, two slower migrating species; for yeast proteasomes these species were identified as complete 26S proteasomes (with RCs capping both ends of the 20S proteasome, i.e., 20S-R C 2) and 26S proteasomes capped only at one end (i.e., 20S-R C 1; Glickman et al., 1998a; Fig. 3 a). The coexistence of two distinct forms of the proteasome has been shown previously by E M (Peters et al., 1993; Fujinami et al., 1994; Walz et al., 1998). To determine which bands contained the RCs, the protein complexes in fraction 19 were electroblotted from the native gels onto nitrocellulose membranes and probed with several mAb bs directed against various subunits of the RC and the 20S proteasome. Fig. 3 c, lane 1, shows immunoreactivity with α3/50, an antibody directed against the RC subunit p39A, and lane 2 shows immunoreactivity with V.DS, an antibody directed against the 20S proteasome. Several other antibodies were used to verify the assignment (data not shown). These results lead to the identification of the four resolved complexes from top to bottom as follows: 26S (20S-R C 2), single 26S (20S-R C 1), RCs, and 20S core particles, as indicated in Fig. 3.

**Subunit Composition of the RC**

As reported previously and illustrated by Fig. 2, Dro-
Drosophila 26S proteasomes are resolved on one-dimensional (1D) gels into 12 distinct bands that are in addition to the bands that arise from the 20S core complex (Udvardy, 1993). A according to their apparent mass, they are referred to as p110 to p37B (Haracska and Udvardy, 1996). All spots marked with a capital were identified by peptide sequencing with the exception of spot L, which could not be sequenced, and spot U, which was identified by immunoblotting (for details see Table I and Results). Low molecular weight proteins (not indicated separately) belong to the 20S core complex. With one exception, Pros35, they were not identified by peptide sequencing.

range that we assume to correspond to the fourth protein, p42A, were not always present and it was not possible to derive sequence information from them. It was also impossible to obtain sequence information for spot L, which showed very faint staining on 2D gels. Since variably migrating forms were found for several subunits (see Fig. 4 and Table I), spot L may correspond to p42D, as do spots J and K. The partially unsatisfactory resolution of the above electrophoretic system prompted the use of another gel system, 16-BA C/SDS-PAGE 2D gel electrophoresis (Hartinger et al., 1996). This gave a better separation, yielding some additional spots. Sequence comparison showed one to correspond to Rpn5/p55; no corresponding spot was found on conventional 2D gels. In the 42-kD region, no additional proteins were detected with the 16-BA C/SDS-PAGE 2D gel system.

A total of 18 distinct RC subunits were identified (Table II). Accordingly, the six ATPases of the AAA family (Conflonieri and Duguët, 1995; Beyer, 1997), which are integral components of all RCs investigated to date (De- Martino et al., 1994; Dubiel et al., 1995; Glickman et al., 1998a), are also present in the Drosophila RC. As their yeast counterparts, the six ATPases from Drosophila embryos were separated in the first dimension by isoelectric focusing (IEF) with an immobilized pH gradient from 3 to 10. Next, proteins were resolved in the second dimension using a 12.5% polyacrylamide SDS gel and stained with Coomassie blue. 26S proteasomes resolved by 1D SD S-PA GE were used as molecular weight marker and the bands were named according to the procedure of Haracska and Udvardy (1996). All spots marked with a capital were identified by peptide sequencing with the exception of spot L, which could not be sequenced, and spot U, which was identified by immunoblotting (for details see Table I and Results). Low molecular weight proteins (not indicated separately) belong to the 20S core complex. With one exception, Pros35, they were not identified by peptide sequencing.

Figure 3. Nondenaturing PAGE of 26S proteasomes. Fractions 10, 13, 16, and 19 (40 μl) from the sucrose gradients were electrophoresed for 800 V·h on 4.5% native polyacrylamide gels. a, Proteolytic activity of the resolved complexes was detected by fluorogenic peptide overlay with Suc-LLVY-AMC and the proteins were visualized by Coomassie blue stain (b, same gel). b, To further characterize the bands, duplicate samples of fraction 19 were transferred to nitrocellulose membranes and immunostained with μ3/50, an mAb directed against the RC subunit, p39A, and with another, V.D5, directed against the 20S proteasome. The analysis of a, b, and c allowed unambiguous identification of the bands as 20S proteasomes, R Cs, 26S proteasomes with only one RC attached (20S-R-C1), and 26S proteasomes with two RCs (20S-R-C2), as indicated.

Figure 4. 2D gel electrophoresis of Drosophila 26S proteasomes. Purified 26S proteasomes from Drosophila embryos were separated in the first dimension by isoelectric focusing (IEF) with an immobilized pH gradient from 3 to 10. Next, proteins were resolved in the second dimension using a 12.5% polyacrylamide SDS gel and stained with Coomassie blue. 26S proteasomes resolved by 1D SD S-PA GE were used as molecular weight marker and the bands were named according to the procedure of Haracska and U dvardy (1996). A ll spots marked with a capital were identified by peptide sequencing with the exception of spot L, which could not be sequenced, and spot U, which was identified by immunoblotting (for details see Table I and Results). Low molecular weight proteins (not indicated separately) belong to the 20S core complex. With one exception, Pros35, they were not identified by peptide sequencing.
human ATPases varies between 64–72% and between 84-88%, respectively. This level of identity is much higher than that of the non-ATPases of the RC, which ranges from 25–44% and 42–71% between Drosophila and yeast and between Drosophila and human, respectively. Similar to the situation in yeast (Glickman et al., 1998a), the non-ATPase subunit, p37B, is an exception since it is 64% identical to yeast Rpn11 and 88% identical to human S13. A iso, p110 (Rpn2/S11) is 25% identical to p97 (Rpn1/S2), and p39B (Rpn8/S12) is 30% identical to p37B (Rpn11/S13).

**Table II. Subunit Composition of the Drosophila RC**

| Name        | Accession* | AM | MW† | pI‡ | Yeast§ Identity| Human§ Identity |
|-------------|------------|----|-----|-----|----------------|-----------------|
| p110        | AF145303   | 1020 | 113.2 | 4.93 | Rpn2 38 (1–858) | S1 64 (3–859)   |
| p97         | AF145304   | 919  | 102.3 | 5.48 | Rpn1 36 (65–615) | S2 58 (65–917)  |
| p58         | M63010     | 494  | 56.0  | 9.04 | Rpn3 32 (12–458) | S3 58 (8–494)   |
| p56         | U39030     | 439  | 49.3  | 6.17 | Rpn2 72 (4–439) | S4 88 (1–439)   |
| p55         | AF145315   | 502  | 57.7  | 5.49 | Rpn5 38 (8–442) | S55 47 (1–455)  |
| p54         | S79502     | 396  | 42.6  | 4.70 | Rpn10 41 (1–225) | S5a 52 (1–381)  |
| p50         | AF145305   | 428  | 47.8  | 5.20 | Rpn5 68 (14–428) | S6 84 (9–428)   |
| p48A        | AF145306   | 412  | 46.9  | 5.22 | Rpn3 69 (34–413) | S6 84 (12–413)  |
| p48B        | AF145307   | 433  | 48.5  | 5.75 | Rpn1 64 (5–433) | S7 85 (1–433)   |
| p42A        | AF145308   | 389  | 45.4  | 6.06 | Rpn7 37 (15–389) | S10 70 (1–380)  |
| p42B        | AF145309   | 422  | 47.3  | 5.66 | Rpn6 42 (1–421) | S9 64 (2–422)   |
| p42C        | U97538     | 405  | 45.8  | 8.51 | Rpn6 71 (18–405) | S8 86 (6–405)   |
| p42D        | AF145310   | 390  | 44.2  | 8.44 | Rpn4 70 (8–388) | S10b 86 (6–390) |
| p39A        | AF145311   | 382  | 43.8  | 5.18 | Rpn9 30 (8–379) | S11 42 (15–381) |
| p39B        | MB6443     | 338  | 38.5  | 8.90 | Rpn8 44 (10–290) | S12 71 (5–290)  |
| p37A        | AF145312   | 324  | 37.7  | 5.12 | UCH37 9 (1–324) | S19 67 (1–329)  |
| p37B        | AF145313   | 308  | 34.4  | 5.74 | Rpn11 64 (1–308) | S13 88 (1–308)  |
| p30         | AF145314   | 264  | 30.2  | 5.80 | Rpn12 25 (25–264) | S14 45 (7–264)  |

*Genbank/EMBL/DDBJ accession number.
†Molecular mass (kD) and pI were theoretically determined by the use of GENETYX-MAC 8.0.
‡Molecular mass and pI of the yeast RC subunits were predicted by the use of GENETYX-MAC 8.0.
§Identities were judged by BLAST searches using default parameters (http://www.ncbi.nlm.nih.gov/blast/psiblast.cgi). The first and last residues of the alignable regions of the Drosophila proteins are given in parentheses.
¶Except for p55 and UCH37, human RC subunits are numbered according to their SDS-PAGE mobility (Dubel et al., 1995).

**p37A, A Novel Subunit of the RC**

One of the spots on the 2D gels yielded the protein sequence FCQCFDPYNK, and sequencing the corresponding EST cDNA clone, LD02040, revealed the existence of an open reading frame of 972 bp, which encodes a previously unreported protein of 324 amino acids, p37A. The predicted molecular mass of p37A, 37.672 kD, matches its SDS-PAGE mobility. The predicted pI of 5.12 is slightly more acidic than that observed on 2D gels (Fig. 4). Drosophila p37A has no homologues among the yeast RC subunits. However, a database search revealed homologues in the human, murine, and bovine genomes, of which the human was identified as a RC subunit (Xu, W., and R.E. Cohen, personal communication). p37A is a member of the ubiquitin COOH-terminal hydrolase (UCH) family (Fig. 5; Wilkinson, 1997). UCHs form a class of thiol proteases, which remove thiols, amines, peptides, and small proteins from the COOH terminus of ubiquitin (Wilkinson, 1997). They are characterized by a 210-amino acid catalytic domain with four highly conserved sequence blocks, containing the four active side residues. Interestingly, p37A is closely related to human BRCA1 (Jensen et al., 1998). BRCA1 is a nuclear protein of 82 kD, showing significant homology to the UCHs in its 240 residue NH2-terminal domain. The COOH-terminal domain of BRCA1 (residues 640–716) is again homologous to p37A and UCH37. Residues conserved among p37A, UCH37, and BRCA1, as well as the other five proteins are shown in reverse type. The active site residues (denoted with arrows) are conserved in all eight proteins.
STEM (Engel et al., 1982) allows such measurements to be made with remarkable accuracy.

Images recorded for mass measurement from unstained 26S proteasome samples showed the preparations to be heterogeneous. Heterogeneity was not reduced by glutaraldehyde fixation. However, the bone-shaped side-view projections, typical of symmetrical 26S (20S-RC2), could be clearly distinguished from the wedge-shaped projections of asymmetrical 26S (20S-RC1) complexes. In addition, there were many ring structures with the characteristic signature of 20S proteasome particles viewed end-on, as well as less dense, almost circular projections. Since their high scattering allowed an unambiguous visual identification, the 20S proteasomes served as a convenient internal mass standard. The mass was set to 721 kD (see Materials and Methods). In this way, both slight mass differences arising from the sample preparation techniques employed, and the inherent beam-induced mass loss, could be accurately accounted for.

Pooled data sets from the four experiments were displayed in histograms and were Gaussian curve fitted (Fig. 6). The low standard deviation of ±55 kD of the 20S proteasome particles (n = 3,853; SE = ± 0.9 kD; Fig. 6 b) illustrates the high quality of the calibration, yielding almost exactly the SD value expected (~50 kD) from background fluctuations for the small selection box size used. The mass of the wedge-shaped species, 20S-RC1, was found to be 1,623 ± 155 kD (n = 2,003; Fig. 6 c). The mass of the 20S-RC2 complexes was 2,508 ± 196 kD (n = 286; Fig. 6 d). The population of small particles, with lower scattering power than the 20S proteasomes' almost circular projections, had a mass of 358 ± 96 kD (n = 1,394; Fig. 6 a). The above data allow three estimates to be made for the mass of the RC: considering the mass of 20S-RC2 and the mass of 20S-RC1, mass of one RC, 1,394; for the small particles with an almost circular projection; b, 721 ± 55 kD for the 20S proteasome top-views (internal mass standard); c, 1,623 ± 155 kD (n = 2,003) for 20S-RC1; and d, 2,508 ± 196 kD (n = 286) for the bone-shaped 26S projections, 20S-RC2.

Deubiquitylating Activity of p37A and the 26S Proteasome

The cDNA encoding p37A was subcloned into pET22b, after fusing a Histidine-tag to its COOH terminus, and expressed in E. coli. The recombinant protein could be purified under native conditions and was assayed for UCH enzymatic activity using U b-A l as a substrate (Dang et al., 1998). Similar to the control enzyme, U CH-L3, p37A hydrolyzed the fluorogenic substrate, and was completely inhibited by U b-A I when added at an equimolar concentration (Fig. 7 a). Purified 26S proteasomes were assayed in the same manner and also found to exhibit U CH enzymatic activity (Fig. 7 b). This activity could be blocked by the addition of U b-A I, but was not affected by the 20S proteasome inhibitor, lactacystin (Fenteany et al., 1995), which rules out the possibility that the 20S core contributes to the release of free AMC.

Mapping the Position of p37A Within the RC by Electron Microscopy

In mapping of p37A, we have taken advantage of the specific binding of the U CH inhibitor, U b-A I. The U b-A I was conjugated to colloidal gold particles with a diameter of 3.5 nm. The gold particles are sufficiently electron dense to be clearly visible, even in negatively stained preparations. To obtain specific and stable conjugates, the side chain of lysine residues of U b-A I was extended by reacting the terminal amino group with 2-iminothiolane to give a modified side chain with a terminal primary thiol group. Stable binding to the colloidal gold particles was then achieved through a covalent gold-sulfur bond.

A micrograph of negatively stained 26S proteasomes, which were labeled with U b-A I conjugated to 3.5-nm colloidal gold particles, is shown in Fig. 8 a. About 50% of the 26S complexes have at least one bound gold particle, whereas unbound gold particles are not detected.
Fig. 8 b shows a gallery of aligned 26S proteasomes carrying a gold particle on either one or both sides of the complex. 2D image analysis (averages obtained after rotational and translational alignment, followed by MSA/classification) clearly map p37A to the interface between the proximal and the distal mass of the RC (Fig. 6 c), i.e., to the interface between the two RC subcomplexes, the base and the lid (Glickman et al., 1998b).

Discussion

Our understanding of the structure and function of the 26S proteasome advances at a relatively slow pace; even establishing the subunit composition is not a trivial task, given the labile nature of the complex. The bovine and human 26S complexes have been investigated in some detail and the primary structures of many of their ~18 electrophoretically distinct subunits in the mass range of 20–110 kD have been reported (D’Emartino et al., 1994; Dubiel et al., 1995; Tanaka and Tsurumi, 1997). For yeast, a set of 18 RC subunits has been described (Glickman et al., 1998a); unfortunately, it is difficult to obtain structurally homogeneous preparations of the complex from yeast. Drosophila embryos have been shown to be a particularly rich source of 26S proteasomes (Udvardy, 1993), and they yield preparations that are sufficiently homogenous for structural studies (Walz et al., 1998). Initial studies with Drosophila RCs identified 12 subunits on 1D gels that were named...
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p110 to p37 (Haracska and Udvardy, 1996). However, complete primary structures have to date only been reported for p54 (Haracska and Udvardy, 1995) and p42C (DUG; Mounkes and Fuller, 1998).

To provide a platform for an in-depth structural analysis of the Drosophila RC, we established the subunit composition of purified 26S proteasomes by 2D gel electrophoresis and subsequent amino acid sequence analysis. In total, 18 subunits were found to constitute a single RC. Of them have homologues among the known yeast and mammalian RC subunits; hence, it can be assumed that none of these proteins is a contaminant and all are integral parts of the Drosophila RC. p37A, the only subunit missing in yeast, has homologues in mammalian 26S proteasomes (Xu, W., and R.E. Cohen, personal communication). The mammalian subunits, S6b (Deveraux et al., 1995) and p28 (Hori et al., 1998), as well as yeast Rpn4 (Fujimoto et al., 1998), have not been consistently found in 26S proteasomes from other organisms. In the Drosophila RC, we found no homologues of these three subunits, in agreement with their absence in Drosophila EST cDNA libraries.

It is hard to assert that the 18 proteins listed in Table II represent the full complement of the Drosophila RC subunits, as subunits may be lost during purification or escape detection on 2D gels. But the close agreement between their summed masses (932 kD) and the experimentally determined mass (894 kD) suggests that the proteins derived from 2D gels indeed represent the complete set of RC subunits from our purified 26S proteasomes. It is interesting to note that in the STEM measurements, the SDs of the mass values were somewhat larger than expected from statistical background fluctuations, with the exception of the 20S particles where the SD is very close to the theoretically expected value. This indicates that the 20S core complex is stoichiometrically well defined, while the R Cs display some heterogeneity. We did not find isolated and structurally well defined particles in the range of intact RCs. Instead, we found a sizeable fraction of roughly spherical particles yielding a broad peak with a maximum at 35B kD. Probably various subcomplexes of the RC (base, lid) contribute to this slightly asymmetric peak.

Most, but not all known RC subunits have been detected in all eukaryotic organisms investigated so far; hence, there seems to be a set of constitutive proteins that are essential for functional 26S proteasomes, and in addition, facultative proteins. The latter may only be expressed in certain organisms or in a tissue-dependent manner at specific developmental stages. Some of these facultative subunits may associate only transiently with the RC, and it will depend critically on the time point when a sample is taken whether they are detected or not. In fact, transient binding to the RC has been reported for Doa4 (Papa et al., 1999) and A p-uch (Hegde et al., 1997), and also the bona fide subunit p54 is present in free and RC-bound form (Haracska and Udvardy, 1995). Therefore, it appears unlikely that a universal number of subunits building the RC can be given.

Upon binding of ubiquitylated protein to the 26S proteasome, ubiquitin is usually recycled by means of deubiquitylating enzymes. Indeed, two different deubiquitylating activities have been reported to occur within the 26S proteasome. A n early study described a 30-kD ubiquitin COOH-terminal hydrolase in the RC of rabbit reticulocytes that cleaves off the remnant of target proteins from the ubiquitin chains in the course of degradation (Eytan et al., 1993). More recently, an isopeptidase activity was found that shortens ubiquitin chains conjugated to target proteins by repeated removal of distal ubiquitins (Lam et al., 1999). Thus, the degradation signal of the protein is removed and poorly or erroneously ubiquitylated proteins may be rescued from proteolysis. A member of the UCH family of deubiquitylating enzymes, UCH 37, is thought to be responsible for this so-called editing activity of the mammalian RC (Xu, W., and R.E. Cohen, personal communication).

We found the homologue of human UCH 37 in Drosophila 26S proteasomes, which we named p37A, and expressed in E. coli for further characterization. Like other recombinant proteins of this family, it cleaves the model substrate Ub-AMC and is inhibited by Ub-Al. Thus, Drosophila p37A is probably different from 30-kD UCH found in proteasomes from rabbit reticulocytes, since the latter is insensitive to Ub-Al (Eytan et al., 1993). We do not know yet what type of conjugates Drosophila p37A prefers as substrates, but like other UCHs, it may cleave ubiquitin from peptides or small protein remnants only (Wilkinson, 1997). However, it has been shown for Drosophila UCH-D (Roff et al., 1996) that UCH activity may not be restricted to small leaving groups. In addition, substrate preferences may differ between the free protein and the protein integrated into the RC. R recombinant UCH 37, for instance, has typical UCH specificity, i.e., it removes an intact ubiquitin chain from a ubiquitin-protein conjugate, whereas UCH 37 embedded in the 19S complex shortens a ubiquitin chain from the distal end by removing ubiquitin moieties one by one, as mentioned above (Xu, W., and R.E. Cohen, personal communication).

Since we found similar enzymatic activity and inhibition profiles with native 26S proteasomes and with recombinant p37A, we assume that p37A is at least in part responsible for the deubiquitylation of proteasome-bound conjugates. The fact that no homologue exists in the yeast genome suggests that p37A is not an essential subunit of the RC, and it cannot be excluded that there are additional subunits that exhibit deubiquitylating activity. Subunit P37B is a candidate that could confer deubiquitylating activity to 26S proteasomes, since it has some sequence similarity to ubiquitin-specific processing proteases (UBP), the second family of deubiquitylating enzymes (Wilkinson, 1997). Whether P37B and its homologues, Rpn4 and S13, possess any enzymatic activity has hitherto not been shown experimentally. Since recombinant Pad1, the P37B homologue in S. pombe, shows no deubiquitylating activity (Penney et al., 1998), it remains questionable whether P37B and its homologues are indeed functional UBP s. Recently, it was shown that a sizeable fraction of Doa4, a 100-kD UBP, copurifies with yeast 26S proteasomes (Papa et al., 1999); however, a Doa4 homologue is not present in our preparations.

Besides p37A and UCH 37, the only other UCH for which an association with 26S proteasomes has been reported is A p-uch. A p-uch is a neuron-specific protein, which is induced during long-term facilitation in Aplysia.
nervous tissue and which binds transiently to 26S proteasomes (Hegde et al., 1997). We did not find an A-uch homologue in our preparations, but could have missed it; because of its small molecular mass, it might be difficult to separate from the 14 subunits of the 20S proteasome. Whether p37A is a constitutive component of the R C, or is only expressed during embryonic stages of the Drosophila development remains to be established. UCH-D, for instance, is only present in high levels during the first four hours of embryogenesis, a rapid decline to low levels follows thereafter (Zhang et al., 1993).

Although a low-resolution three-dimensional (3D) map of the Drosophila 26S proteasome exists (Walz et al., 1998), the mapping of specific subunits to this structural framework is only in its beginnings. There is evidence from biochemical and genetic studies that the six A T Pases, all members of the A A A - A T Pase superfamily, are closely associated with each other; by way of analogy to other members of the A A A - A T Pase family, it has been inferred that the six paralogs form a heterohexameric ring (Voges et al., 1999). Supposing that they act as reverse chaperones, unfolding substrates before their translocation into the 20S proteolytic core, they were tentatively mapped to the interface between 20S core and the remainder of the R C (Lupas et al., 1993). Recentley a combined genetic, biochemical, and structural approach has provided more definitive insights into the structural organization of the R C (Glickman et al., 1999c). In yeast, the R C has been dissected into two distinct subcomplexes, the base and the lid. The base, which is proximal to the 20S core complex, indeed comprises the six A T Pases and, in addition, the two largest subunits, R p n 1 and R p n 2. The eight remaining subunits were assigned to the lid. The interaction between the base and the lid is destabilized by deletion of the subunit R p n 10. Therefore, it can be assumed that R p n 10 is critically involved in providing a structural linkage. Otherwise, the role of R p n 10 is enigmatic: it binds mult ubiquitin chains in vitro (Deveraux et al., 1994), but is dispensable in vivo for the degradation of ubiquitylated proteins (Van Nocker et al., 1996). This could be reconciled if one assumes that other subunits, probably in the lid, are responsible for the initial binding of ubiquitylated proteins, while R p n 10 stabilizes the interaction further downstream in the process.

Having identified p37A as a bona fide component of the Drosophila R C, we have mapped its location by E M. To this end, we have taken advantage of the specific binding of the U CH inhibitor U b - A I to its target. By coupling U b - A I to 3-nm colloidal gold particles, a strong signal was generated that was clearly visible, even on unprocessed electron micrographs. On averaged images, the U b - A I gold conjugates map to the neck region of the dragon-head motif, i.e., the hinge between the base and the lid. This is the region where we also assume that R p n 10 is located (see above). Thus, it appears that both the binding of multi ubiquitin chains and deubiquitylation are spatially closely related and perhaps also functionally coupled. One could envisage a scenario in which ubiquitylated proteins initially bind to the lid subcomplex. While being transferred to the base where the substrate is prepared for its feeding into the 20S core, R p n 10 prevents its escape while p37A recycles bound ubiquitin.

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References

Baumeister, W., B. Dahlmann, R. Hegeli, F. Kopp, L. Kuehn, and G. Pfeifer. 1988. Electron microscopy and image analysis of the multicytologic proteinase. FEBS Lett. 241:239-245.

Baumeister, W., J. Walz, F. Zuhl, and E. Seeemerler. 1998. The proteasome: paradigm of a self-compartmentalizing protease. Cell. 92:3673-3680.

Beyer, A. 1997. Sequence analysis of the A A A protein family. Protein Sci. 6:2043-2058.

Bochtil, M., L. Ditilzi, M. Groll, C. Hartmann, and R. Huber. 1999. The proteasome. Annu. Rev. Biophys. Biomol. Struct. 28:295-317.

Braun, B. C., M. Glickman, R. Kraft, B. Dahlmann, P. Kloeetzell, D. Finley, and M. Schmidt. 1999. The base of the proteasome regulatory particle exhibits chaperone-like activity. Nat. Cell Biol. 1:1221-1226.

Confalonieri, F., and M. Duguet. 1995. A 200-amino acid AT Pase module in the interface between 20S core and the remainder of the R C (Voges et al., 1999). Recentley a combined genetic, biochemical, and structural approach has provided more definitive insights into the structural organization of the R C (Glickman et al., 1999c). In yeast, the R C has been dissected into two distinct subcomplexes, the base and the lid. The base, which is proximal to the 20S core complex, indeed comprises the six A T Pases and, in addition, the two largest subunits, R p n 1 and R p n 2. The eight remaining subunits were assigned to the lid. The interaction between the base and the lid is destabilized by deletion of the subunit R p n 10. Therefore, it can be assumed that R p n 10 is critically involved in providing a structural linkage. Otherwise, the role of R p n 10 is enigmatic: it binds mult ubiquitin chains in vitro (Deveraux et al., 1994), but is dispensable in vivo for the degradation of ubiquitylated proteins (Van Nocker et al., 1996). This could be reconciled if one assumes that other subunits, probably in the lid, are responsible for the initial binding of ubiquitylated proteins, while R p n 10 stabilizes the interaction further downstream in the process.

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References

Baumeister, W., B. Dahlmann, R. Hegeri, F. Kopp, L. Kuehn, and G. Pfeifer. 1988. Electron microscopy and image analysis of the multicytologic proteinase. FEBS Lett. 241:239–245.

Baumeister, W., J. Walz, F. Zuhl, and E. Seeumerler. 1998. The proteasome: paradigm of a self-compartmentalizing protease. Cell. 92:3673–3680.

Beyer, A. 1997. Sequence analysis of the A A A protein family. Protein Sci. 6:2043–2058.

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Braun, B. C., M. Glickman, R. Kraft, B. Dahlmann, P. Kloeetzell, D. Finley, and M. Schmidt. 1999. The base of the proteasome regulatory particle exhibits chaperone-like activity. Nat. Cell Biol. 1:1221–1226.

Confalonieri, F., and M. Duguet. 1995. A 200-amino acid AT Pase module in the interface between 20S core and the remainder of the R C (Voges et al., 1999). Recentley a combined genetic, biochemical, and structural approach has provided more definitive insights into the structural organization of the R C (Glickman et al., 1999c). In yeast, the R C has been dissected into two distinct subcomplexes, the base and the lid. The base, which is proximal to the 20S core complex, indeed comprises the six ATPases and, in addition, the two largest subunits, Rpn1 and Rpn2. The eight remaining subunits were assigned to the lid. The interaction between the base and the lid is destabilized by deletion of the subunit Rpn10. Therefore, it can be assumed that Rpn10 is critically involved in providing a structural linkage. Otherwise, the role of Rpn10 is enigmatic: it binds mult ubiquitin chains in vitro (Deveraux et al., 1994), but is dispensable in vivo for the degradation of ubiquitylated proteins (Van Nocker et al., 1996). This could be reconciled if one assumes that other subunits, probably in the lid, are responsible for the initial binding of ubiquitylated proteins, while Rpn10 stabilizes the interaction further downstream in the process.

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Hegerl, R. 1996. The EM program package: a platform for image processing in biological electron microscopy. J. Struct. Biol. 116:30–34.

Herschko, A., and A. Ciechanover. 1998. The ubiquitin system. Annu. Rev. Biochem. 67:425–479.

Hoffman, L., G. Pratt, and M. Rechsteiner. 1992. Multiple forms of the 20S multicatalytic and the 26S ubiquitin/A TP-dependent proteases from rabbit reticulocyte lysate. J. Biol. Chem. 267:22362–22368.

Horii, T., S. Kato, M. Saeki, G.N. DeMartino, C.A. Slaughter, J. Takeuchi, A. Toh-e, and K. Tanaka. 1998. CDA cloning and functional analysis of p28 (Nas6p) and p40.5 (Nas7p), two novel regulatory subunits of the 26S proteasome. Gene. 216:113–122.

Hough, R., G. Pratt, and M. Rechsteiner. 1987. Purification of two high molecular weight proteases from rabbit reticulocyte lysate. J. Biol. Chem. 262:8303–8313.

Jensen, D.E., M. Proctor, S.T. Marquis, H.P. Gardner, S.I. Ha, L.A. Chodosh, J. Peters, J.M., Z. Cejka, J.R. Harris, J.A. Kleinschmidt, and W. Baumeister. 1993. Structural features of the 26S proteasome complex. J. Mol. Biol. 234:932–937.

Larsen, C.N., and D. Finley. 1997. Protein translocation channels in the proteasome. Mol. Biol. Cell. 8:1079–1112.

Laemmli, U.K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227:680–683.

Lam, Y.A., G.N. DeMartino, C.M. Pickart, and R.E. Cohen. 1997a. Specificity of the ubiquitin isopeptidase in the PA 700 regulatory complex of 26S proteasomes. J. Biol. Chem. 272:28438–28446.

Lam, Y.A., W. Xu, G.N. DeMartino, and R.E. Cohen. 1997b. E editing of ubiquitin conjugates by an isopeptidase in the 26S proteasome. Nature 385:737–740.

Larsen, C.N., and D. Finley. 1997. Protein translocation channels in the proteasome and other proteases. Cell. 91:431–434.

Lupas, A., M. Van Dyke, and J. Stock. 1991. Predicting coiled coils from protein sequences. Science 252:1162–1164.

Lupas, A., A.J. Koster, and W. Baumeister. 1993. Structural features of 26S and 20S proteasomes. Enz. Prot. 47:252–273.

Lupas, A., J.M. Flanagan, T. Tamura, and W. Baumeister. 1997. Self-compartmentalizing proteases. Trends Biochem. Sci. 22:299–304.

Munkes, L.C., and M.T. Fuller. 1998. The DUG gene of Drosophila melanogaster encodes a structural and functional homolog of the Saccharomyces cerevisiae SGU1 predicted ATPase associated with the 26S proteasome. Gene. 206:165–174.

Müller, S.A., N.G. Kenneth, R. Bürki, R. Häring, and A. Engel. 1992. Factors influencing the precision of quantitative scanning transmission electron microscopy. Ultramicroscopy. 46:317–334.

Papa, F.A., A.Y. A merik, and M. Hochstrasser. 1999. Interaction of the Drosophila deubiquitinating enzyme with the yeast 26S proteasome. Mol. Biol. Cell. 10:741–756.

Patterson, S.D. 1994. From electrophoretically separated protein to identification: strategies for sequence and mass analysis. An. Biochem. 221:1–15.

Penney, M., C. Wilkinson, M. Wallace, J.P. Javerzat, K. Ferrell, M. Seeger, W. Strickland, E., K. Hakala, P.J. Thomas, and G.N. DeMartino. 2000. Recognition of misfolding proteins by PA 700, the regulatory subcomplex of the 26S proteasome. J. Biol. Chem. 275:5565–5572.

Peters, J.M., P. Zwickl, and A. Steven. 2000. Dis-assembly lines: the proteasome, activates protein breakdown by 20S proteasomes. J. Biol. Chem. 275:28438–28446.

Rubin, D.M., and D. Finley. 1995. The proteasome: a protein-degrading organelle? Curr. Biol. 5:854–858.

Rubin, G.M., L. Hong, P. Brokstein, M. Evans-Holm, E. Frise, M. Stapleton, and D.A. Harvey. 2000. A Drosophila complementary DNA resource. Science. 287:2222–2224.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: a laboratory Manual. 2nd. edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Scheffner, M., S. Smith, and S. Jentsch. 1998. The ubiquitin-conjugation system. In Ubiquitin and the Biology of the Cell. J.M. Peters, J.R. Harris, and D. Finley, editors. Plenum Press, N.Y. 65–98.

Slot, J.W., and H. Geuze. 1985. A new method of preparing gold probes for multiple-labeling cytochemistry. Eur. J. Cell Biol. 38:87–93.

Strickland, E., K. Hakala, F.J. Thomas, and G.N. DeMartino. 2000. Identification of misfolding proteins by PA 700, the regulatory subcomplex of the 26S proteasome. J. Biol. Chem. 275:5565–5572.

Studier, F.W., A.H. Rosenberg, J.J. Dunn, and J.W. Dubendorf. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. 185:60–89.

Tanaka, K., and C. Tsurumi. 1997. The 26S proteasome: subunits and functions. Mol. Biol. Rep. 24:3–11.

Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin, and D.G. Higgins. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. 25:4876–4882.

Ueda, R., A. 1993. Purification and characterization of a multisubunit component of the Drosophila 26S (1500 kD) proteolytic complex. J. Biol. Chem. 268:9055–9062.

Van Noeker, S., S. Sadis, D.M. Rubin, G. Glickman, H. Fu, O. Coux, I. Wefes, D. Finley, and R.D. Vierstra. 1996. The multibiquitin-chain-binding protein Mcbl is a component of the 26S proteasome in Saccharomyces cerevisiae and plays a nonessential, substrate-specific role in protein turnover. Mol. Cell. Biol. 16:6020–6028.

Varshavsky, A. 1997. The ubiquitin system. Trends Biochem. Sci. 22:383–387.

Voges, D., P. Zwickl, and W. Baumeister. 1999. The 26S proteasome: a molecular machine designed for controlled proteolysis. Annu. Rev. Biochem. 68:1005–1018.

Wält, A., A. Erdmann, M. Kania, D. Typke, A. J. Koster, and W. Baumeister. 1999. 26S proteasome structure revealed by 3-dimensional electron microscopy. J. Struct. Biol. 121:19–29.

Wenzel, T., and W. Baumeister. 1995. Conformational constraints in protein degradation by the 20S proteasome. J. Struct. Biol. 2:189–204.

Wilkinson, K.D. 1997. Regulation of ubiquitin-dependent processes by deubiquitinating enzymes. FA SE B J. 11:1245–1256.

Wolf, S., I. Nacy, A. Lupas, G. Pfeifer, Z. Cejka, S.A. Müller, A. Engel, R. De Mot, and W. Baumeister. 1998. Characterization of A RC, a divergent member of the AAA ATPase family from Rhodococcus erythropolis. J. Mol. Biol. 277:13–25.

Yoshimura, T., K. Kameyama, T. Takagi, A. Ikai, F. Tokunaga, T. Koida, N. Tanahashi, T. Tamura, Z. Cejka, W. Baumeister, et al. 1993. Molecular characterization of the 26S proteasome complex from rat liver. J. Struct. Biol. 111:200–211.

Zhang, N., K.D. Wilkinson, and M. Bownes. 1993. Cloning and analysis of expression of a ubiquitin carboxyl terminal domain protease expressed during oogenesis in Drosophila melanogaster. Dev. Biol. 157:214–223.

Zwickl, P., D. Nig, K. Min Woo, H.P. Klenk, and A.L. Goldberg. 1999. An archaeabacterial ATPase, homologous to ATPases in the eukaryotic 26S proteasome, activates protein breakdown by 20S proteasomes. J. Biol. Chem. 274:26008–26014.

Zwickl, P., W. Baumeister, and A. Steven. 2000. Dis-assembly lines: the proteasome and related ATPase-associated proteases. Curr. Opin. Struct. Biol. 10:242–250.