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Dissection of the Regulator Complex of the *Drosophila* 26S Protease by Limited Proteolysis

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The 26S protease responsible for the selective degradation of ubiquitinated proteins is composed of a regulator complex and the 20S proteosome which is the catalytic core. In the absence of ATP the 26S protease dissociates to free regulator complex and 20S proteosome, and this process can be reversed in vitro in the presence of ATP. Trypsin, chymotrypsin or proteinase K digestion selectively removes several subunits of the free regulator complex of *Drosophila* 26S protease generating a well-defined new subparticle. Three subunits highly sensitive in the free regulator complex, however, were selectively protected within the in vitro reconstituted 26S protease, indicating that the ATP-dependent association of the 20S proteosome to the regulator complex selectively shields these subunits. In the same concentration range the 20S proteosome was completely resistant for proteolytic degradation.

In eukaryotic cells the ubiquitin pathway and the 26S protease is responsible for the controlled proteolysis of cytosolic and nuclear proteins. The ubiquitin pathway marks proteins intended for rapid intracellular degradation by the covalent attachment of ubiquitin, and these tagged proteins are specifically recognised and cleaved by the 26S protease (for most recent reviews see 1–9). This large multiprotein enzyme is composed of the 20S proteosome and the regulator complex. The 20S proteosome, a multicatalytic protease capable of cleaving peptide bonds carboxy-terminal to basic, acidic and hydrophobic amino acids (7,10) is the catalytic core of the 26S protease. Its proteolytic activity is ATP independent, and it has no any specificity towards ubiquitinated proteins. This specificity is ensured by the regulator complex. Digital image analysis of negatively stained electron microscopic pictures revealed that the regulator complex is cap-shaped, and after ATP-dependent reconstitution two cap-shaped regulator complexes are attached end-on to the bases of the cylindrical 20S proteosome (11,12). Although the 20S proteosome is the catalytic core of the 26S protease, the binding of the regulator complexes to the 20S proteosome substantially change its enzymatic properties: proteolysis by the 26S protease is absolutely ATP-dependent and it is confined to ubiquitinated proteins (13–16). The ATP hydrolysis which accompanies the binding of the regulator complex to the 20S proteosome and the proteolytic process is probably maintained by the ATPase subunits of the regulator complex (17–21), while another subunit of the regulator complex with high binding affinity for multiubiquitinated proteins ensures probably the specificity towards ubiquitinated proteins (22).

In *Drosophila melanogaster* the regulator complex is composed of 15 subunits ranging in size from 37 to 110 kDa (15). The conserved ATPase domain is present on subunits p48 and p42b (18).

MATERIALS AND METHODS

Purification of the 20S proteosome and the regulator complex, and in vitro reconstitution of the 26S protease was described earlier (15). To study the limited proteolysis of the regulator complex the partially purified DEAE-cellulose fraction (15) was digested with increasing concentrations of trypsine (Sigma), chymotrypsine (Sigma) or proteinase K (Merck) in buffer A (20 mM Hepes pH 7.6, 100 mM NaCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol and 5% glycerol) at 25 °C for 60 min. The reaction was terminated by loading the samples onto a Superose 6 prep grade column (Pharmacia)

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equilibrated with buffer A and fractionating as described earlier (15). For limited proteolysis of the 26S protease the enzyme was in vitro reconstituted by preincubating the partially purified DEAE-cellulose fraction in the presence of ATP and an ATP-regenerating system (15), and the trypsine, chymotrypsine or proteinase K digestion was done after the reconstitution as described above. The 26S protease or its proteolytic derivatives were finally purified by Superose 6 chromatography in buffer A + 0.3 mM ATP. The effect of partial proteolysis on the subunit structure of the regulator complex or the 26S protease was studied by silver staining or immunoblotting. As the elution position of the proteolytically modified free regulator complex was shifted towards the lower molecular weight region, where several other multiprotein complexes elute, the change in the subunit structure of the regulator complex can be more specifically demonstrated by immunoblot analysis. SDS-polyacrylamide gel electrophoresis, silver staining and immunoblotting were performed by standard procedures (24). The polyclonal antibody specific for the *Drosophila* regulator complex was described earlier (15). Polyclonal antibody specific for 20S proteosome subunits was risen in rabbits by standard procedures (24).

RESULTS AND DISCUSSION

The regulator complex of the Drosophila 26S protease and the 20S proteosome can be separated by size fractionating a partially purified DEAE-cellulose fraction of a *Drosophila* embryonic

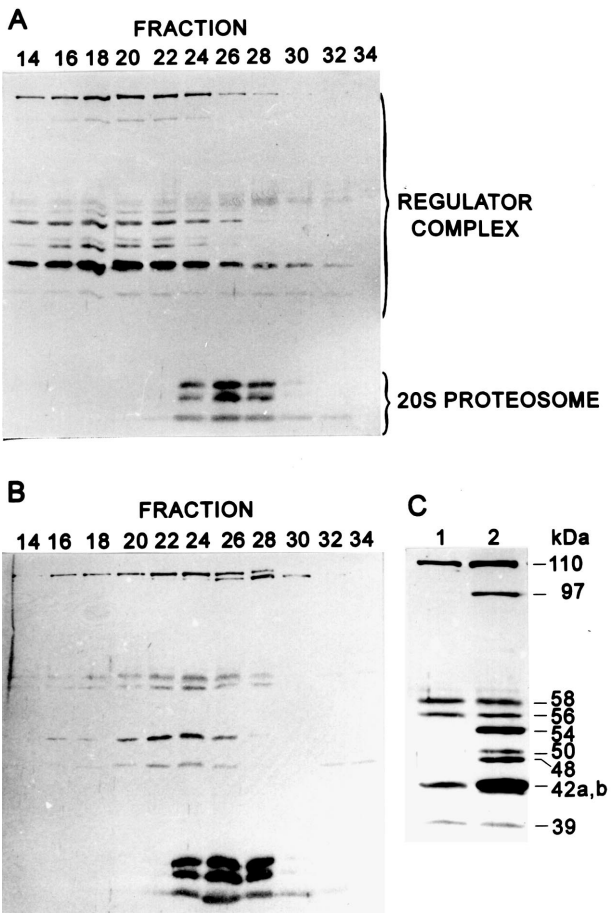

FIG. 1. Characterisation of the subparticle derived from the free regulator complex by trypsin digestion. (A) DEAE-cellulose fraction of a *Drosophila* embryonic extract was fractionated on Superose 6 column. Immunoblot analysis of the chromatography fractions using a mixture of anti-regulator complex and anti-20S proteosome antibodies. The analysed chromatography fractions are indicated on the top of the figure. Subunits belonging to the regulator complex or the 20S proteosome are marked on the right side of the figure. (B) DEAE-cellulose fraction preincubated with 0.6 μg/ml trypsin was fractionated and analysed as described in A. (C) Comparison of the subunit pattern of the trypsin digested (lane 1, fraction 24 from B) and intact (lane 2, fraction 20 from A) regulator complex.

protein extract on a Superose 6 column (15). As shown in Fig. 1A the regulator complex and the 20S proteosome elutes around fractions 20 and 26, respectively. After trypsine digestion of the partially purified DEAE-cellulose fraction the elution position of the regulator complex was shifted towards the lower molecular weight region, while no change was observed in the elution position of the 20S proteosome (Fig. 1B). Immunoblot analysis of the Superose 6 chromatography fractions performed with a mixture of anti-regulator complex and anti-20S proteosome antibodies revealed that p97, p54, p50, p48 and p42b subunits of the regulator complex were attacked by trypsine digestion, while the rest of the subunits of the regulator complex were left intact (Fig. 1). Removal of these subunits generated a new, well-defined subparticle of the regulator complex which elutes around fraction 24 (Fig. 1B). As shown on Fig. 2 the generation of this new subparticle occurred over a trypsine concentration range of 0.024–1.0 μg/ml. Subunits p54 and p42b are the most susceptible for proteolytic cleavage. The shift in the elution position on Superose 6 column occurs immediately after the removal of subunits p54 and p42b, when all the other subunits seem to be intact (data not shown), suggesting an exposed configuration of these subunits on the regulator complex. Similar proteolytic degradation pattern can be induced by chymotrypsine or proteinase K digestion (data not shown). The 20S proteosome is much more resistant against proteolytic degradation. Neither its subunit composition nor its elution position changed in the trypsine concentration range effective in reshaping the regulator complex (Fig. 1B). The high protease resistance of the *Drosophila* 20S proteosome is in good agreement with previously published data on mice 20S proteosome (25).

p50, p48, and p42b subunits of the regulator complex are selectively protected against proteolysis in the reconstituted 26S protease. The 26S protease can be reconstituted in vitro by preincubating the partially purified DEAE-cellulose fraction in the presence of ATP and an ATP-regenerating system (15). The reconstituted 26S protease is a 2:1 stoichiometric complex of the regulator complex and the 20S proteosome, and due to its larger mass it elutes ahead of the free regulator complex on a Superose 6 sizing column around fraction 17 (15). To study the proteolytic sensitivity of the subunits of the regulator complex within the reconstituted 26S protease, the partially purified DEAE-cellulose fraction was preincubated in the presence of ATP and an ATP-regenerating system, followed by digestion with increasing concentrations of trypsine. The modified 26S protease was purified on Superose 6 column in buffer A + 0.3 mM ATP, and its subunit pattern was analysed by silver staining. As shown on Fig. 3 the proteolytic degradation pattern of

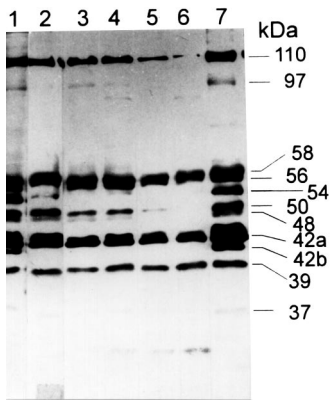

FIG. 2. Trypsin sensitivity of the subunits of the free regulator complex. DEAE-cellulose fraction was incubated with increasing concentrations of trypsin, fractionated on Superose 6 column and fraction 22 from each chromatography was analysed by immunoblotting using anti-regulator complex antibody. Lane 1: undigested sample; lane 2: 0.024 μg/ml trypsin; lane 3: 0.12 μg/ml trypsin; lane 4: 0.3 μg/ml trypsin; lane 5: 0.6 μg/ml trypsin; lane 6: 1 μg/ml trypsin; lane 7: undigested sample.

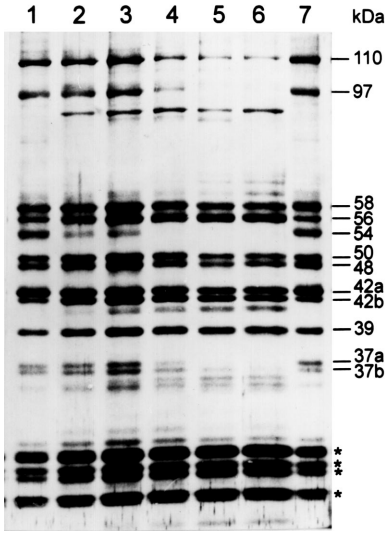

FIG. 3. Trypsin sensitivity of the subunits of the regulator complex within the reconstituted 26S protease. DEAE-cellulose fraction was preincubated in the presence of ATP and an ATP-regenerating system, followed by digestion with increasing concentrations of trypsin. The 26S protease was purified on Superose 6 prep grad column and fraction 18 from each chromatography was analysed by gel electrophoresis and silver staining. Lane 1: undigested sample; lane 2: 0.01 μg/ml trypsin; lane 3: 0.02 μg/ml trypsin; lane 4: 0.1 μg/ml trypsin; lane 5: 0.5 μg/ml trypsin; lane 6: 1 μg/ml trypsin; lane 7: undigested sample. Subunits of the 20S proteosome are marked by asterisk.

the regulator complex within the reconstituted 26S protease is basically changed. p54 and p97 are by far the most susceptible subunits to proteolysis. p54 is almost completely removed from the regulator complex at 0.1 μg/ml trypsine concentration where p97 is the only other subunit seriously cleaved (Fig. 3 lane 4). The accessibility of trypsine to subunits p50, p48 and p42b is drastically reduced. On Superose 6 sizing column there is only a minor shift in the elution position of the proteolytically modified 26S protease lacking subunits p97, p54 and p37, it elutes around fraction 18 (data not shown).

From the proteolytic degradation pattern certain conclusions can be drawn for the shape of the free regulator complex and for the arrangement of different subunits within the reconstituted 26S protease. p54 is the most susceptible for proteolysis within the free regulator complex (Fig. 2), suggesting that it is in an exposed position. Previously we have shown that p54 is the only subunit of the regulator complex which is present in significant concentration not only as a particle-bound, but also as a free polypeptide in *Drosophila* embryos. Indirect evidences suggested that it is a shuttling subunit, exchanging between free-and particle-bound form (23). The exposed location of p54 within the regulator complex supports this assumption.

Subunits p50, p48 and p42b should be in an exposed position in the free regulator complex as they are readily degraded by trypsine, but they should be buried in the 26S protease. This may either be due to a rearrangement in the higher order structure of the regulator complex during the reconstitution of the 26S protease, or more probably to a physical shielding of these subunits by the 20S proteosome. This later assumption is supported by the observation that subunits p54, p97 and p37 were removed in the same order from the free regulator complex or the 26S protease, indicating that no gross rearrangement occurred at least in the region of these subunits. Thus, it is reasonable to suppose that subunits p50, p48 and p42b are on that strand of the V-shaped regulator complex which attaches to the base of the 20S proteosome (11,12). Two of these subunits, p48 and p42b, carry the conserved ATPase domain (18), which are most probably involved in the ATP-dependent association of the regulator complex and the 20S proteosome. The core of the regulator complex-

composed of subunits p58, p56, p42a and p39 - should be a very compact particle which is highly resistant against proteolytic attack.

The elution position of a multiprotein complex on Superose 6 sizing column depends both on the mass and the shape of the complex. There is a sharp shift in the elution position of the free regulator complex after limited proteolytic modification, suggesting a change not only in the mass, but also in the shape of the particle. As the elution position of the free regulator complex after mild trypsine digestion which removes only subunits p97, p54, p42b and p37 is shifted to fraction 24 during Superose 6 chromatography (Fig. 2), these subunits may contribute significantly to the asymmetric shape of the regulator complex.

As the proteolytic degradation pattern of the free regulator complex or the reconstituted 26S protease is very similar with three different proteases, including proteinase K with very low sequence specificity, the shape of these particles and not the distribution of protease recognition sequences may be responsible for the specificity of cleavages.

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