Involvement of proteasomal subunits zeta and iota in RNA degradation

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We have identified two distinct subunits of 20 S proteasomes that are associated with RNase activity. Proteasome subunits zeta and iota, eluted from two-dimensional Western blots, hydrolysed tobacco mosaic virus RNA, whereas none of the other subunits degraded this substrate under the same conditions. Additionally, proteasomes were dissociated by 6 M urea, and subunit zeta, containing the highest RNase activity, was isolated by anion-exchange chromatography and gel filtration. Purified subunit zeta migrated as a single spot on two-dimensional PAGE with a molecular mass of approx. 28 kDa. Addition of anti-(subunit zeta) antibodies led to the co-precipitation of this proteasome subunit and nuclease activity. This is the first evidence that proteasomal α-type subunits are associated with an enzymic activity, and our results provide further evidence that proteasomes may be involved in cellular RNA metabolism.

INTRODUCTION

The sub-ribosomal fractions of eukaryotic cells contain a number of protein complexes with a relatively high sedimentation rate. Most abundant are the 20 S proteasomes, which associate with other particles to form larger complexes. One of these, the 26 S proteasome, has attracted much attention during the last few years [1–3], as it has the ability to degrade protein–ubiquitin conjugates in an ATP-dependent reaction [4,5]. Furthermore, proteasomes are involved in cell cycle control [6,7], in early steps of the immune response [8,9] and in transcriptional regulation [10,11], and recently published data suggest that proteasomes may also participate in the pathways of cellular RNA breakdown [12,13].

Proteasomes have a cylindrical structure. The cylinder consists of a stack of four discs or rings, each of which is composed of seven subunits with molecular masses of between 19 and 35 kDa. Two-dimensional protein gel electrophoresis has revealed up to 20 individual proteins, with the number varying between cells and species [14–20]. In addition, proteasomes were reported to contain about 0.0016–0.2 % low-molecular-mass RNAs. The RNA content depends on the origin of the proteasomes, and decreases with increasing proteasome purity [13,21]. These RNAs are heterogeneous in size, but migrate most frequently in a molecular size range of 70–120 nucleotides [22–24]. Partial sequence analysis of proteasomal RNA has revealed that no particular species of RNA is specifically associated with the proteasomes [21]. Thus proteasomal RNAs were suggested to represent contamination of purified proteasomes [21] or residual substrate fragments of a specific proteasomal RNase activity [12,25]. The latter hypothesis is attractive, since it has been shown that nuclease activity is an integral part of proteasomes. Using different types of chromatography, Pouch et al. [12] have shown that RNase activity was coincident with the elution profiles at each state of purification, and that nuclease activity was resistant to strong dissociation conditions using 0.5 % lauroylsarkosyl-Na and 6 M urea.

In the present paper, we demonstrate that two proteasome α-type subunits are responsible for RNA hydrolysis. Subunit zeta showed the most rapid cleavage reaction, whereas subunit iota was less active. No other proteasome subunits tested degraded tobacco mosaic virus (TMV) RNA under the same conditions.

EXPERIMENTAL

Definition of TBK buffers

Buffer TBK X consists of 20 mM Tris/HCl, pH 7.0, X mM KCl, 2.5 mM $MgCl_2$ and 7 mM 2-mercaptoethanol, where X is 0, 50, 120, 240, 300 or 480.

Cell fractionation and isolation of proteasomes from calf liver cells

The preparation of the post-mitochondrial supernatant has been described [26]. Proteasomes were isolated from the post-mitochondrial supernatant by several ultracentrifugation steps. The final purification was by FPLC (Pharmacia) using Mono Q and Superose 6 columns, as reported previously [12].

Assay of RNA digestion

Purified TMV RNA from *Nicotiana rustica* was purchased from DSM (Braunschweig, Germany). A portion of 4 μg of TMV RNA was incubated for 20 min with or without proteasomes or proteasome proteins at 37 °C. The volume of each assay was 200 μl, and the incubation buffer was TBK 120. After incubation, the assays were applied to a Superose 6 column equilibrated in TBK 120 containing 3 mM EDTA. The percentage degradation of RNA was calculated by measuring the peaks of absorbance at 280 nm before and after digestion.

Protein gel electrophoresis

Proteins were precipitated with 10 % (w/v) trichloroacetic acid (final concentration). Electrophoresis of proteins was performed

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on one-dimensional SDS/polyacrylamide gels [27]. Two-dimensional gel electrophoresis was performed according to [28] using Ampholines (Pharmacia) in the pH range 3.5–10. For the first dimension, the gels were run at 400 V for 12 h, and then, for the second dimension, at 800 V for 1 h. Molecular mass markers were phosphorylase *b* (94 kDa), BSA (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soy bean trypsin inhibitor (20 kDa) and lactalbumin (14 kDa).

Immunoblotting

After electrophoresis, the proteins were transferred to a nitrocellulose membrane using a Trans-Blot SD Semi-Dry Transfer Unit (Bio-Rad) in a buffer containing 25 mM Tris/HCl, pH 8.3, 192 mM glycine and 20 % (v/v) methanol. After 1 h of transfer, the membrane was blocked for at least 1 h in 5 % (w/v) non-fat dried milk in TTBS buffer [20 mM Tris/HCl, pH 7.4, 500 mM NaCl and 0.1 % (v/v) Tween 20]. The membrane was then washed for 3×15 min at 37 °C in TTBS before incubation with one of the monoclonal antibodies (IB5, purchased from Cappel; or MCP196) diluted 1:2000 in TTBS overnight at room temperature. After washing in TTBS, the membrane was incubated with alkaline phosphatase-labelled anti-mouse IgG antibodies diluted 1:2000 in TTBS for 1 h at 37 °C. The membrane was then washed again, and alkaline phosphatase-conjugated antibodies were detected by the Nitro Blue Tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate system [29].

Immunoprecipitation

The mouse IgG anti-(subunit zeta) antibody MCP196 was purified from mouse peritoneal fluid using an FPLC Protein A–Superose column (Pharmacia) [29]. Purified 28 kDa protein was incubated with anti-(subunit zeta) antibody [30] for 2 h at room temperature. Then Protein A–Sepharose CL-4B (Pharmacia) was added (5 % final concentration) and the incubation continued for 1 h with gentle agitation at room temperature. After 5 min of centrifugation (10000 *g*; Hettich Mikroliter), the supernatant was subjected to SDS/PAGE or assayed for RNase activity.

Recovery of proteasome subunits from electroblots

Proteasomal proteins were separated by two-dimensional PAGE and transferred to nitrocellulose sheets by semi-dry blotting. The different subunits were visualized by Ponceau Red. Then the stained spots were cut out, and proteins were eluted from the membrane in 200 μl of TBK 240 containing 10 % (v/v) acetonitrile and 0.2 % (w/v) Triton X-100 for 5 h at 37 °C [31].

RESULTS

Localization of RNase activity on proteasome subunits

Previously published data indicated that the integrity of the proteasome complex is not necessary for RNase activity [12]. Thus we assumed that dissociation of proteasomes could represent a useful method for the isolation of proteasomal subunits that hydrolyse RNA.

In a preliminary series of investigations, purified proteasomes were dissociated by 8 M urea and subunits were separated by two-dimensional PAGE (Figure 1). After transfer to nitrocellulose sheets, individual proteasomal subunits were eluted with buffers containing acetonitrile and Triton X-100 [31] and then tested for RNase activity. Staining of nitrocellulose sheets with Ponceau Red before and after elution revealed that approx. 80 %
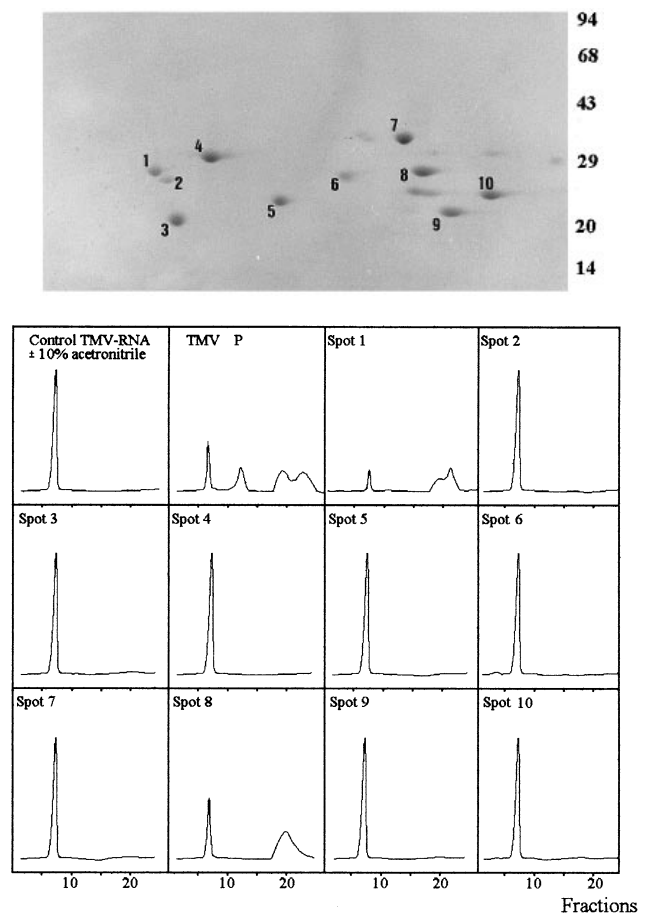

Figure 1   Screening of proteasome subunits for RNase activity

Proteasomes subunits were separated by two-dimensional PAGE and transferred to nitrocellulose sheets. Ponceau Red-stained protein spots (upper panel) were cut out and eluted from the membrane with TBK 240 containing 10% acetonitrile and 0.2% Triton X-100 (see the Experimental section). The eluted fractions (200 μl) were incubated with 4 μg of TMV RNA for 20 min at 37 °C, and then the total assay was analysed by gel filtration on Superose 6 columns (lower panels). The percentage degradation of RNA was calculated as described in the Experimental section: spot 1/subunit zeta, 70–80%; spot 8/subunit iota, 30–40%; other numbered spots, 0–2%. P indicates total proteasome. Absorbance at 280 nm is shown. Proteasomal subunits iota and zeta were identified by immunoblotting using the monoclonal antibodies MCP196 (zeta) and IB5 (iota). The numbers to the right of the upper panel indicate molecular mass (kDa)

of transferred proteins were recovered (results not shown). To demonstrate RNase activity we used as a substrate TMV RNA, a polycistronic messenger with an apparent molecular mass of about 2000 kDa, which separates well from 20 S proteasomes (700 kDa) on gel-filtration columns ([12]; see the Experimental section). These experiments revealed that the major activity was associated with subunit zeta, while lower activity was detected when subunit iota was present (Figure 1). None of the other proteasomal subunits eluted from the nitrocellulose sheets cleaved TMV RNA under the same conditions (Figure 1).

Purification of subunit zeta associated with RNase activity

To confirm our findings, we decided to purify subunit zeta, as it had the highest RNA cleavage activity. In this approach, proteasomes were dissociated in urea and the subunits were separated by ion-exchange chromatography on Mono Q. Proteasomal subunits were eluted in the range 100–300 mM KCl with
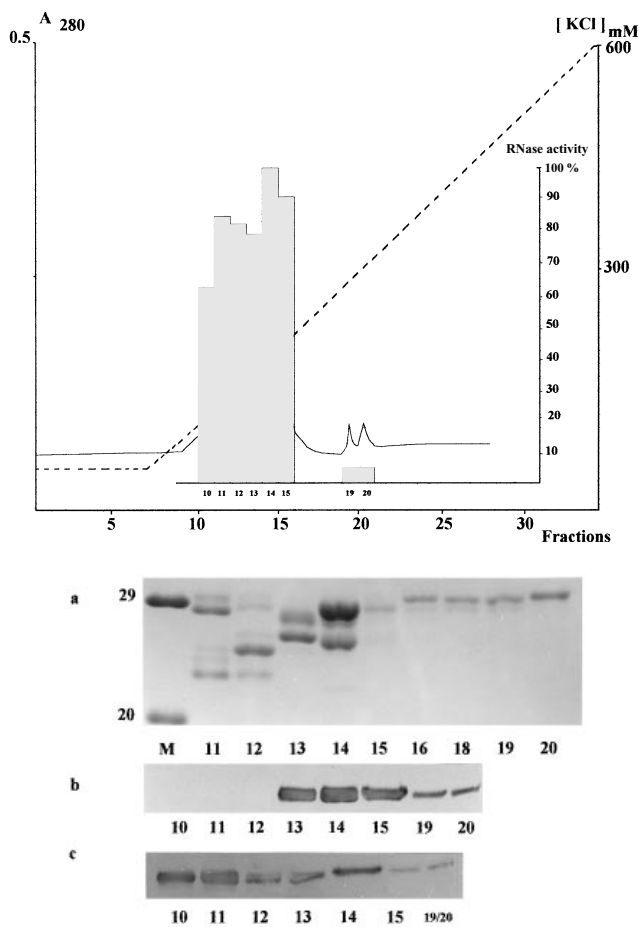
Figure 2   Co-elution of RNase activity with proteasomal subunits

Top panel: 2 mg of highly purified proteasomes eluted from Superose 6 columns was dialysed against TBK 0, and then incubated for 10 min at 37 °C with 6 M urea (final concentration). The suspension was loaded on to an FPLC Mono Q HR 5/5 column equilibrated in TBK 0 containing 6 M urea. Proteasomal subunits were eluted with a linear salt gradient containing 6 M urea from TBK 0 to TBK 600. Eluted fractions were dialysed against TBK 120, and 200 μl aliquots were incubated with 4 μg of TMV RNA for 20 min at 37 °C to analyse RNase activity, as described in the Experimental section. RNase activity is shown by the bar diagram, the solid line indicates A_{280}, and the broken line indicates KCl concentration. (**a**–**c**) Distribution of subunits zeta and iota in fractions eluted from FPLC Mono Q columns. Portions of 800 μl of fractions 11–20 (top panel) were incubated with 10% trichloroacetic acid (final concentration) to precipitate the proteins. Sedimented proteins were analysed by PAGE. After gel electrophoresis, proteins were transferred to nitrocellulose filters and probed with the monoclonal antibodies MCP196 and IB5. Mouse IgG was detected as described in the Experimental section. (**a**) Proteasome subunits visualized by Coomassie Blue staining (lane M contains molecular mass markers; kDa); (**b**) subunit zeta detected by MCP196; (**c**) subunit iota detected by IB5.

Figure 3   Analysis by gel filtration of fraction 14 eluted from the FPLC Mono Q column

Upper panel: fraction 14 obtained by Mono Q chromatography (Figure 2) was applied on an FPLC Superdex 75 column equilibrated in TBK 120 containing 6 M urea. Eluted fractions were dialysed against TBK 120 to eliminate urea. Samples (200 μl of the 1 ml fractions 5–9) were incubated with 4 μg of TMV RNA for 20 min at 37 °C to assay RNase activity. RNase activity is shown by the bar diagram; the line indicates A_{280}. Lower panel: for PAGE, eluted fractions were pooled (lane a, 5 + 6; lane b, 7; lane c, 8 + 9) and dialysed against TBK 120 to eliminate urea. Samples (800 μl) of the pooled fractions were incubated with 10% (w/v) trichloroacetic acid (final concentration) to precipitate the proteins. Sedimented proteins were analysed by SDS/PAGE and the gels were stained with Coomassie Blue. Lane M contains molecular mass markers (kDa).

a heterogeneous profile of absorbance (Figure 2, top panel). PAGE of the eluted fractions revealed that most fractions contained more than one proteasomal subunit (Figure 2a). Two proteasomal proteins larger than 29 kDa were eluted separately in the range 150–300 mM KCl from the bulk of proteasomal subunits and contained only very low RNase activity (Figure 2, top panel, fractions 19 and 20; and Figure 2a, lanes 18–20).

The major part of the RNase activity co-eluted with the bulk of proteasome subunits, and analysis by immunoblotting with antibodies MCP 196 and IB5 (Figures 2b and 2c) demonstrated that all fractions eluted from the Mono Q column contained at least one of the two subunits exhibiting RNase activity, i.e. zeta and iota. Only in fractions 10–12 was subunit zeta completely
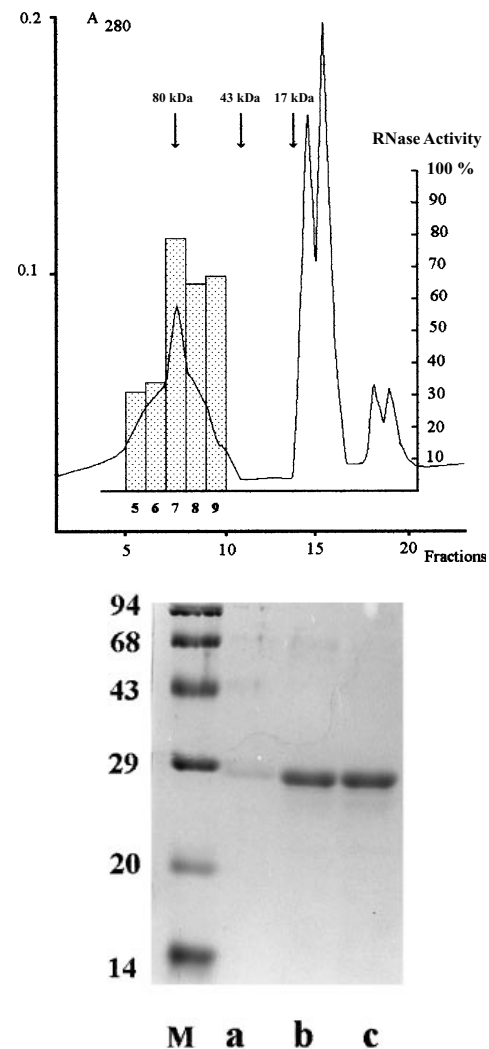
absent. Thus treatment of proteasomes with 6 M urea and subsequent chromatography on Mono Q only partially dissociates the 20 S complex.

For further purification of the RNase-containing proteasome subunit, fraction 14 from Mono Q chromatography, which had the highest RNase activity (Figure 2, top panel) and the highest subunit zeta content (Figure 2a), was analysed further by gel filtration on a Superdex 75 column (Figure 3, upper panel). To our surprise, all protein co-eluted with RNase activity in fractions 5–9, corresponding to a molecular mass range of 40–90 kDa, and PAGE revealed that these fractions contained exclusively
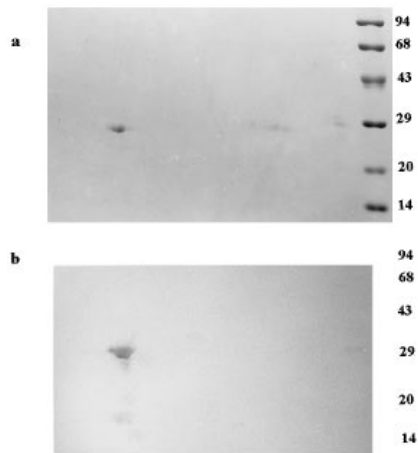
Figure 4   Two-dimensional PAGE analysis of purified subunit zeta

Subunit zeta of proteasomes purified by gel filtration on FPLC Superdex 75 (see Figure 3) was analysed by two-dimensional PAGE, and then the proteins were transferred to a nitrocellulose sheet and probed with antibody MCP 196, as indicated in the Experimental section. (**a**) Coomassie Blue staining of purified subunit zeta; (**b**) immunoblot of purified subunit zeta detected with antibody MCP 196. Positions of molecular mass markers (kDa) are indicated on the right.

Figure 5   RNase activity of proteasome subunit zeta and subunit of 32 kDa

Proteasome subunit zeta eluted from FPLC Superdex 75 columns (see Figure 3), as well as C2 purified from fraction 20 of Figure 2, were dialysed against TBK 120 to eliminate urea and incubated with TMV RNA for 20 min at 37 °C. Subsequently the total assay (200 μl) was immediately analysed by chromatography on FPLC Superose 6 equilibrated in TBK 120. (**a**) TMV RNA (4 μg) was incubated with 200 μl samples of fractions containing purified proteasome subunit zeta. (**b**) A suspension of subunit zeta in TBK 120 was incubated for 2 h with monoclonal antibody MCP196 in the presence of Protein A–Sepharose. Immunocomplexes bound to Protein A–Sepharose were sedimented by centrifugation and 200 μl of the supernatant was assayed for RNase activity for 20 min at 37 °C (4 μg of TMV RNA) or subjected to PAGE [inset: lane 1, subunit zeta (control); lane 2, supernatant after immunoprecipitation; lane M, molecular mass markers]. (**c**) Fraction 20 eluted from FPLC Mono Q columns (Figure 2) and containing the largest proteasome subunit C2 were subjected to gel filtration on Superdex 75 columns under the same conditions as described in the legend to Figure 3. Eluted fractions showing the highest absorbance at 280 nm were dialysed against TBK 120 to eliminate urea. Then they were incubated with 10% (w/v) trichloroacetic acid (final concentration) to precipitate the proteins. Sedimented proteins were analysed by SDS/PAGE (inset). Alternatively, 200 μl portions of the dialysed fractions were tested for RNase activity by gel filtration as described above.

proteasome subunits with a molecular mass of 28 kDa (Figure 3, lower panel). We concluded that these subunits are α-type subunits that were eluted as oligomeric aggregates under the conditions employed. Indeed, such self-aggregation was also described for α-type subunits expressed in bacteria, whereas proteasomal β-type subunits, which are all smaller than 27 kDa, do not polymerize [32a]. This would explain the good separation by gel filtration of α-type from β-type subunits with quite similar molecular masses (Figure 2b, lane 14; Figure 3, lower panel).

For further analysis, fractions 5–9 containing the 28 kDa protein (see Figure 3) were subjected to two-dimensional PAGE. Figure 4(a) demonstrates that the 28 kDa protein migrated as a single spot. Comparison of the protein pattern of intact 20 S proteasomes (Figure 1) with the migration of the 28 kDa protein on two-dimensional gels (Figure 4a) shows that the 28 kDa subunit is the most acidic protein, corresponding to subunit zeta of the 20 S proteasome complex [30]. Figure 4(b) demonstrates that the 28 kDa protein is actually recognized by monoclonal antibody MCP196, which is specific for subunit zeta. In addition, incubation of a suspension of purified 28 kDa protein with MCP 196 led to complete precipitation of this proteasomal subunit (Figure 5b, inset, lane 2), and the remaining supernatant was free of RNase activity (Figure 5b). From these experiments, we conclude that the RNase activity of 20 S proteasomes is associated with subunit zeta.

Additionally, we tested the largest proteasome subunit, C2, for RNase activity. This proteasomal subunit is known to dissociate easily from the 20 S proteasome complex; it is absent from urea-resistent proteasome subcomplexes that still harbour RNase activity [12]. For purification, material in fraction 20 of several runs on FPLC Mono Q columns was pooled (Figure 2a) and subjected to gel filtration on an FPLC Superdex 75 column under the same conditions as described for the purification of subunit zeta (Figure 3). Analysis of the eluted fractions by PAGE showed that this proteasomal subunit migrated as single band of approx. 32 kDa (Figure 5c, inset). This subunit did not hydrolyse TMV RNA under the conditions employed (Figure 5c).
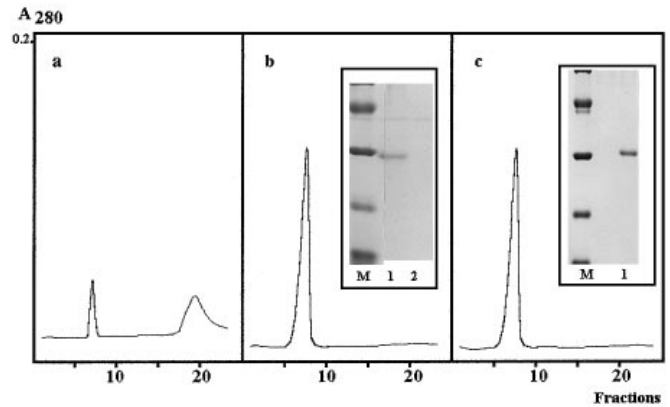
DISCUSSION

Previous reports have shown that proteasomes are completely dissociated by treatment with SDS or bivalent ions such as Zn^{2+} [33]. Incubation with mercurial chloromercuribenzoic acid led to the formation of a heterogeneous population of proteasomal subcomplexes and subunits, as analysed by PAGE [34]. In addition, reverse-phase chromatography was shown to be a powerful tool for the isolation of distinct proteasomal subunits [35]. However, under these conditions the proteolytic activities of proteasomes were abolished.

Here we show that incubation of proteasomes with 6 M urea is a valuable method for the dissociation of proteasomes into subcomplexes and subunits that retain RNase activity. It should be noted that, under these conditions, not only the RNase activity, but also the proteasomal chymotrypsin-like activity, is preserved (J. Buri, F. Petit, Y. Briand and H. P. Schmid, unpublished work). For the isolation of individual proteasome subunits, it was necessary to carry out all chromatographic procedures in the presence of 6 M urea to avoid partial or total reconstitution of proteasomes. Nevertheless the purified 28 kDa subunit of proteasomes showed a high tendency to polymerize, even in the presence of high concentrations of urea, since it was eluted from gel-filtration columns in fractions corresponding to molecular masses of up to 90 kDa. We took advantage of this aggregation of α-type subunits to separate them by gel filtration from non-polymerizing β-subunits. Interestingly, the purified

28 kDa protein had an approx. 10-fold higher nuclease activity than the same amount (A_{280} units) of intact proteasomes. This confirms our findings, shown in Figure 1, that just two of the various proteasome subunits eluted from nitrocellulose sheets contained this activity. All proteolytic activities of proteasomes investigated so far have been associated with the β-type subunits, and the role of the α-type subunits has remained speculative (reviewed in [36]). α-Type subunits act as linkers between the 19 S regulator complex and the 20 S proteasome to build up the 26 S proteasome [37]. However, as clearly demonstrated here, some α-type subunits, such as subunit zeta, are also associated with the expression of an enzymic activity, namely RNase. Subunit iota, another α-type subunit of proteasomes, also degrades TMV RNA, but is less active than subunit zeta. Accordingly, it is possible that the two individual subunits form separate nucleolytic centres. In this respect subunit iota, which was reported to contain an RNA binding domain [38], could be implicated in the recognition of RNA substrates. Work is in progress to purify subunit iota to homogeneity for further investigations.

Our work supports recent findings [12] that RNase activity is an integral part of proteasomes, indicating the existence of a close relationship between proteasomes and RNA. In this context, 20 S proteasomes (prosomes) were detected as factors that control translation in Friend virus-infected mouse erythroblasts by transient association with untranslatable free mRNA [19]. Indeed, several experimental findings support the idea that proteasomes could act as rather selective repressors of translation, as they inhibit *in vitro* protein synthesis of mRNA from adenovirus-infected HeLa cells and the translation of polycistronic TMV RNA, whereas the translation of HeLa mRNA and globin mRNA is not affected under the same conditions [39]. Previous work has shown that proteasomes interfere with the initiation of protein synthesis and prevent the formation of 80 S initiation complexes. TMV RNA cannot associate with the 40 S ribosomal subunits, due to the RNase activity of proteasomes [12,40].

How proteasomes select mRNAs for degradation remains unknown. We assume that sequences or secondary structures exist that are recognized by 20 S proteasomes. Work on mRNA decay has shown that sequence elements such as the 3′ untranslated region regulate the degradation of different eukaryotic messengers. The best studied examples are the iron-response element stem–loop structures on transferrin mRNA and the ARE (see below), which are present in many unstable mRNAs. While the association of a 90 kDa protein with the iron-response element stabilizes transferrin mRNA, its dissociation favours the binding of destabilizing factors. These factors could be ribonucleases or proteins that may recruit ribonucleases to destroy transferrin mRNA [41,42]. The second well characterized mRNA-destabilizing sequence is an AUUUA-rich region within the 3′ untranslated region, named the ARE [43]. Characteristic classes of ARE-containing mRNAs include cytokine, lymphokine and oncogene mRNAs [41]. Among the proteins that associate specifically with the ARE, a 20 S protein complex with unknown protein composition was described and found to be involved in the modulation of the stabilization of ARE elements [44]. Interestingly, 20 S proteasomes and this 20 S protein complex share common properties, e.g. similar size, association with mRNAs and destabilization of RNAs. In addition, preliminary experiments in our laboratory indicate that proteasome-associated RNase activity cleaves AUUUA-rich oligo-RNAs specifically (A.-S. Jarrousse, unpublished work). Future work will show whether proteasomes and this 20 S protein complex are analogues or homologous structures.

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