The Human Proteasomal Subunit HsC8 Induces Ring Formation of Other α -Type Subunits

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The eukaryotic 20 S proteasome is a barrel-shaped protease complex, made up of four seven-membered rings. The outer and inner rings contain seven different α and β -type subunits, respectively, each subunit located at a defined position. Recently, we have reported that the recombinant human α -type subunit C8 (HsC8) assembles into a heptameric ring-like structure by itself. In the present study we show that the two naturally neighboring α -type subunits of HsC8, HsPROS30 and HsPROS27, do not form ring-like complexes by themselves, but only dimers. This indicates that the propensity to form homo-oligomeric rings is not a general feature among human α -type subunits. However, co-expression of HsC8 and either of these neighbor α -type subunits results in the formation of hetero-oligomeric ring complexes, resembling the HsC8 ring-like structure. The ratio between the two types of subunits in the mixed complexes is surprisingly heterogeneous, varying from very high to very low HsC8 content. The three tested α -type subunits thus apparently lack binding sites that selectively interact with a specific neighboring subunit. This suggests that the correct positioning of the different α -type subunits in the eukaryotic 20 S proteasome is not dictated by the α -type subunits themselves, but rather by the interaction with specific β -type subunits.

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Introduction

In eukaryotes, the 20 S proteasome is the major non-lysosomal protease, which can form together with the 19 S regulatory complex, the 26 S proteasome. The 26 S complex is responsible for the ubiquitin-dependent turnover of cytosolic and nuclear proteins (Coux *et al.*, 1996; Hochstrasser, 1995; Peters, 1994). This proteasome-ubiquitin pathway has an essential function in the cell, being involved in processes such as cell cycle progression (Hilt & Wolf, 1996), antigen presentation (Coux *et al.*, 1996) and apoptosis (Grimm *et al.*, 1996; Sadoul *et al.*, 1996). The 20 S proteasome is a multicatalytic enzyme with at least five different protease activities (Rivett, 1993). The complex comprises 28 subunits, arranged in a stack of four seven-membered rings, which form a barrel-shaped structure of about 700,000 M_r (Koster *et al.*, 1995). A relatively simple 20 S proteasome was isolated from the archaeobacterium *Thermoplasma acidophilum*, consisting of only two different subunits, named α and β (Dahlmann *et al.*, 1989). In contrast, several differ-

ent but related eukaryotic proteasomal subunits have been characterized, which can be distinguished into two groups, α and β -type, based on their similarity to either the α - or β -subunit of *T. acidophilum* (Hilt & Wolf, 1996; Scherrer & Bey, 1994). The α -subunits reside in the two outer rings, and the β -subunits form the two inner rings of the 20 S proteasome (Groll *et al.*, 1997; Kopp *et al.*, 1993, 1995, 1997; Löwe *et al.*, 1995; Pühler *et al.*, 1992; Schauer *et al.*, 1993). Importantly, the different α and β -subunits in the eukaryotic proteasome are not randomly distributed within the respective rings, but occupy defined positions, resulting in an $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ complex (Chen & Hochstrasser, 1996; Groll *et al.*, 1997; Kopp *et al.*, 1993, 1995, 1997; Schauer *et al.*, 1993). Thus, the 20 S proteasome can be regarded as a complex dimer with a symmetrical arrangement of 2×14 different subunits along the cylinder main axis (Kopp *et al.*, 1993).

The assembly of the eukaryotic proteasome particle is as yet poorly understood. Assembly intermediates of 13 to 16 S, containing at least six

different α -type subunits and several unprocessed β -subunits, have been documented (Frentzel *et al.*, 1994; Patel *et al.*, 1994; Schmidtke *et al.*, 1997; Yang *et al.*, 1995). Processing of some pro- β -subunits takes place in the 16 S complex, during the final formation of the active 20 S complex (Chen & Hochstrasser, 1996; Frentzel *et al.*, 1994; Schmidtke *et al.*, 1996, 1997). The assembly of 13 to 16 S complexes into the 20 S complex is a relatively slow process, and translation dependent (Frentzel *et al.*, 1994; Yang *et al.*, 1995). In *T. acidophilum* the formation of α -rings is most likely the first step in proteasomal assembly (Zwickl *et al.*, 1994). A box of approximately 25 amino acid residues at the N terminus, highly conserved in all α -type subunits, is essential for this ring formation. Subsequently, the α -rings chaperone the folding, assembly and autocatalytic processing of the β -type subunits into the mature *T. acidophilum* proteasome (Seemüller *et al.*, 1996; Zwickl *et al.*, 1992, 1994).

Like the α -subunit of *T. acidophilum*, the recombinant human α -type subunit C8 (HsC8) forms a double ring structure by itself (Gerards *et al.*, 1997). The role of HsC8 in the assembly process is, however, different from that of the *T. acidophilum* α -subunit, since HsC8 does not form 20 S complexes with recombinant human β -subunits (Gerards *et al.*, 1997). In this study we investigated whether HsC8 has a role in the assembly of different α -type subunits into the ring complex. We show that the naturally neighboring subunits of HsC8 in the human 20 S proteasome (Groll *et al.*, 1997; Kopp *et al.*, 1997), HsPROS30 (Silva-Pereira *et al.*, 1992) also called HsC2 and HsPROS27 (Bey *et al.*, 1993) also called HsIota, do not occur as rings but form dimers *in vitro*. However, upon coexpression with HsC8 these α -type subunits assemble into hetero-oligomeric ring structures. These ring-like complexes have a very variable subunit composition, which indicates that the three analyzed α -type subunits do not have a preference for a specific neighboring subunit. This result suggests that the β -type rather than the α -type subunits play an important role in the correct positioning of the α -type subunits in the 20 S proteasome.

Results and Discussion

Not all recombinant human α -type subunits form ring complexes

To test whether the ability of HsC8 to form ring structures is a general feature of human α -type subunits, we analyzed the assembly properties of HsPROS30 and HsPROS27, which are both naturally neighboring subunits of HsC8 in the human 20 S proteasome (Groll *et al.*, 1997; Kopp *et al.*, 1997). The subunits were produced from pET-expression constructs (Table 1) in *Escherichia coli* BL21(DE3) (Studier *et al.*, 1990). SDS-PAGE analysis of total lysates of bacteria expressing HsPROS30, HsPROS27 or HsC8 (see also (Gerards *et al.*, 1997)) reveals that upon induction the bac-

Table 1. Characteristics of the α -type proteasomal subunits studied

Subunit	Expression construct	Molecular mass (kDa)	pI
HsPROS30	pET3cHs30	30	6.6
HsPROS27	pET3cHs27	27.5	6.7
HsC8	pET16bHsC8, pET24dHsC8	28.5	5.1

Molecular masses and pI were calculated from the predicted amino acid sequence encoded by the expression constructs, using the Pepstats program of the CAOS/CAMM computer facility of the University of Nijmegen.

teria produce a large amount of the recombinant proteins (Figure 1a). These proteins have the correct monomeric size and react with the proper antibodies (Figure 1b), thus confirming that they are the expected proteasomal subunits. The water-soluble fraction of bacteria expressing the proteasomal subunits was isolated for molecular mass estimation. More than 80% of each recombinant subunit was in the water-soluble fraction. On a Superose 12 size exclusion column, both HsPROS30 and HsPROS27 are eluted at the dimer position (Figure 1c). A similar result was obtained on a Superdex 75 column (data not shown). Thus, in contrast with HsC8, which forms a double ring complex (Gerards *et al.*, 1997) of 550 kDa (Figure 1c), neither HsPROS30 nor HsPROS27 subunits by themselves are able to form such complexes. This is most likely not due to aberrant folding of the latter two proteins, as indicated by their good solubility and lack of degradation products in the bacterial lysates. This means that the property of HsC8 to form rings by itself is not a general feature of human α -type subunits.

HsPROS30 and HsPROS27 are incorporated in large complexes upon coexpression with HsC8

Since HsPROS30 and HsPROS27, in contrast to HsC8, do not form ring structures by themselves, we envisaged that the HsC8 complex might play an important role in the assembly of the eukaryotic proteasomal α -ring. HsC8, of which presumably only one subunit is present in each α -ring of the mature proteasome, may form a scaffold to which the other α -type subunits are joined. Therefore, we assessed whether HsPROS30 and HsPROS27, which are both naturally neighboring subunits of HsC8 in the proteasome (Groll *et al.*, 1997; Kopp *et al.*, 1997), occur in higher molecular mass complexes upon coexpression with HsC8 in *E. coli*. The water-soluble proteins of bacteria coexpressing HsC8 and HsPROS30, or HsC8 and HsPROS27, were first fractionated on a Sepharose Q (SQ) anion-exchange column (Figure 2). HsPROS30 and HsC8 are eluted in a broad peak at slightly different salt concentrations (Figure 2a and b). The first peak fractions consist mainly of HsPROS30 (fractions 3 to 5), whereas the last

Figure 1. Expression in *E. coli* and size determination of human α -type proteasomal subunits. **a**, Total bacterial lysates of bacteria expressing the indicated proteasomal subunit, two hours after induction with IPTG. The lysates were separated by SDS-PAGE and stained with Coomassie brilliant blue. The control lane contains the lysate of IPTG-induced bacteria with an empty pET3c vector. The asterisks indicate the positions of the recombinant proteasomal proteins. Molecular masses of the markers (M) are indicated in kDa. **b**, Western blot of total bacterial lysates, as in **a**, and human proteasomes after SDS-PAGE and probing with the monoclonal antibodies, as indicated above each two lanes, directed against the relevant proteasomal subunits. **c**, Complex size of the recombinant α -type subunits. The water-soluble proteins of bacteria expressing HsC8, HsPROS30 or HsPROS27 were fractionated on a Superose 12 size exclusion column. The fractions were analyzed on Western blots which were probed with the relevant monoclonal anti-proteasomal subunit antibodies. Arrows indicate the positions of the peak fraction of marker proteins: thyroglobulin (670 kDa), BSA (67 kDa) and chymotrypsinogen A (25 kDa).

peak fractions contain almost exclusively HsC8 (fractions 9 to 13). The HsC8:HsPROS30 ratio changes gradually between the two extremes. Similarly, when HsPROS27 is coexpressed with HsC8 and fractionated on a Sepharose Q column followed by analysis by SDS-PAGE, both proteins are eluted from the column as a broad peak (not shown) in which the ratio varies gradually (Figure 2c).

To examine the effect of coexpression with HsC8 on the complex size of HsPROS30, we subjected the Sepharose Q fractions 4, 7 and 9 to size exclusion chromatography. Interestingly, in all three fractions HsPROS30 is eluted together with HsC8 in a single peak as large complexes with molecular masses of 575 up to 605 kDa (Figure 3). The apparent molecular mass of the complexes is slightly larger than that of the pure HsC8 complex (about 560 kDa) which corresponds with the difference in molecular mass between the HsPROS30 subunit and the HsC8 subunit. A similar result was

obtained with HsPROS27 coexpressed with HsC8 (data not shown). Thus, contrary to single expression of HsPROS30 or HsPROS27, resulting in apparent dimers, both these α -type subunits are present in a much larger complex upon coexpression with HsC8.

HsC8 induces ring formation of HsPROS30

The size of the HsC8-HsPROS30 and HsC8-HsPROS27 complexes suggests that they may exist as a pair of heptameric rings similar to the HsC8 complex. EM-analysis of the three different HsC8-HsPROS30-containing peaks in Figure 3 (top) confirms that all observed complexes consist of ring-like structures (Figure 4). They have a diameter of $14(\pm 1)$ nm and are indistinguishable from pure HsC8 rings (see Gerards *et al.*, 1997). Side-view images of the complexes appear as two parallel-oriented rod-like structures in all three electron micrographs

Figure 2. Fractionation of bacterial extracts coexpressing HsC8 and HsPROS30 or HsC8 and HsPROS27. a, Sepharose Q anion-exchange chromatography of the bacterial extract containing HsC8 and HsPROS30. Graphs represent the applied salt gradient (broken line) and the obtained elution profile (continuous line). A similar pattern was obtained when a bacterial extract containing HsC8 and HsPROS27 was applied (not shown). b, Coomassie brilliant blue-stained protein pattern after SDS-PAGE of Sepharose Q fractions from HsC8 + HsPROS30. c, Western blot after SDS-PAGE of Sepharose Q fractions containing HsC8 + HsPROS27, probed with the monoclonal antibodies MCP72 and p27k. Lane i contains the input, and the numbered lanes correspond with the fractions in a.

(arrows), indicating that the complexes consist of two stacked heptameric rings. Based on the similarity of HsC8 and HsC8-HsPROS30 complexes with respect to molecular mass, diameter and EM-structure, the mixed ring complexes probably also contain 2×7 subunits.

HsC8-HsPROS30 rings are hetero-oligomers containing at least one HsC8 subunit

The above experiments do not reveal whether the ring particles exist as hetero-oligomeric complexes, composed of a mixture of the two different α -type subunits or, alternatively, as two different homo-oligomeric complexes, each containing a single type of α -subunit. To distinguish between these two possibilities, immunoprecipitations were performed on the HsC8-HsPROS30-containing Sepharose Q fractions 4, 7 and 9 with a polyclonal antiserum raised against HsC8 (Figure 5). Using this serum, HsPROS30 is not precipitated from bacterial lysates expressing HsPROS30 alone, but it does coprecipitate *via* HsC8 from the fractions 4, 7 and 9. The HsC8:HsPROS30 ratio of input and precipitate is very similar, indicating that most if not all of the rings contain both HsC8 and HsPROS30. The formation of stable mixed complexes with different HsC8 and HsPROS30 ratios was also confirmed by rechromatography of the three Sepharose Q fractions on the same column. Proteasomal subunits of each fraction are eluted in a single peak, at a salt concentration corresponding with

the initial run on the column (data not shown). Also HsC8-HsPROS27 complexes containing completely different ratios of the two proteins could be precipitated *via* HsC8 (data not shown). Thus, HsC8 and at least either one of its naturally neighboring subunits can form hetero-oligomeric complexes with a similar molecular mass as the HsC8 complex, in which the ratio between the two proteins is extremely variable.

The existence of complexes consisting mainly of HsPROS30 indicates that two HsPROS30 subunits can be neighbors in a ring. Therefore, it is peculiar that single expressed HsPROS30 does not form ring structures. Incubation of separately expressed HsC8 rings and HsPROS30 results in the binding of only a small amount of HsPROS30 to the HsC8 rings (data not shown). The exchange of subunits between ring complexes is a very inefficient process, which is very likely to be caused by the high stability of the complex (data not shown). This means that HsPROS30 will probably not be exchanged with the α -type subunits already located in the ring and that preformed dimeric HsPROS30 does not have the proper conformation for ring formation upon HsC8 addition. Coexpression with HsC8 thus seems to be essential for efficient ring formation of HsPROS30. A possible role for HsC8 might be that a single monomeric HsC8 subunit, or a HsC8 subunit at either end of an incomplete ring, can bind HsPROS30 and keep it in a conformation that allows another α -type subunit to join. This would imply that each complex

Figure 3. HsPROS30 assembles into large complexes upon coexpression with HsC8. Three of the Sepharose Q fractions shown in Figure 2b, containing mainly HsPROS30 (fraction 4) or HsC8 (fraction 9) or an equal amount of both subunits (fraction 7), were applied to a Superose 6 size exclusion column. The upper panel shows the elution profiles. Arrows indicate the peak positions of the marker proteins, thyroglobulin (670 kDa) and ferritin (440 kDa), used to estimate the indicated molecular masses. Peak-fractions were analyzed on SDS/polyacrylamide gels stained with Coomassie brilliant blue (lower panel); i is the input.

contains at least one HsC8 subunit. To investigate this, Sepharose Q fraction 4 was depleted of HsC8 by three sequential immunoprecipitation steps

with anti-HsC8. The final supernatant still contains a small amount of HsPROS30 (less than 5% of the input) but almost no HsC8 (Figure 6a). The remain-

Figure 4. Electron micrographs of HsC8-HsPROS30 complexes. Panels show the negatively stained complexes in the peak fractions of the three Superose 6 separations of HsC8 and HsPROS30 depicted in Figure 3 (top panel), representing fractions 4, 7 and 9 of the Sepharose Q column. Arrows indicate side views of double ring structures. The scale bar represents 100 nm.

Figure 5. HsC8 and HsPROS30 form mixed complexes. The Sepharose Q fractions 4, 7 and 9 (see Figure 2b), and as a control the water-soluble fraction of single expressed HsPROS30, were immunoprecipitated with an anti-HsC8 polyclonal antiserum. The input and precipitates (ip) were analyzed by non-reducing SDS-PAGE and subsequent Coomassie brilliant blue staining of the gels. As a control a pre-immune serum was used.

ing HsPROS30 is eluted from a Superose 6 column at the dimer position (Figure 6b), like the single expressed HsPROS30. The apparent absence of large homo-oligomeric HsPROS30 complexes indicates that at least one HsC8 subunit is present in all HsPROS30 ring complexes, suggesting that HsC8 is essential for the assembly of the hetero-oligomeric ring complexes.

The composition of the α -rings may be regulated by the β -type subunits

Our data show that the composition of complexes consisting of two α -type subunits is extremely variable. The ratio of HsPROS30 and HsC8,

and also of HsPROS27 and HsC8, in the ring complex may vary between 1:6 and 6:1, and all combinations in between. Thus, in these ring complexes, both HsPROS30 and HsPROS27 can have itself or HsC8 as neighbor subunit on both sides. This flexibility seems in contrast with the fact that in the eukaryotic 20 S proteasome each α -type subunit occupies a given position with two defined neighbor subunits (Kopp *et al.*, 1993, 1995; Schauer *et al.*, 1993). Our results clearly show that the tested α -type subunits do not contain a binding site for a specific neighboring α -type subunit. To reconcile these distinct observations we conjecture that the β -type subunits have an important role in the correct positioning of subunits in the mature α -ring. This would be in agreement with the finding that in the crystal structure of the yeast 20 S proteasome especially α - β and β - β contacts are very specific (Groll *et al.*, 1997). If β -type subunits determine the correct positioning of the α -type subunits, it probably is too simple an approach to try and understand the assembly of the eukaryotic proteasome by extrapolating the model for the *T. acidophilum* proteasome, which starts with the formation of a complete α -ring, followed by the binding of the β -type subunit precursors. It is more likely that the assembly of the α and the β -type subunits in the eukaryotic proteasome occurs in a coordinate fashion; a given α -type subunit binds to a defined β -type subunit which in turn determines the next α -type subunit to bind, and so on. This is in accordance with the finding that the 13 to 16 S proteasomal intermediates contain both α and β -type subunits (Frentzel *et al.*, 1994; Patel *et al.*, 1994; Yang *et al.*, 1995). In the eukaryotic assembly process, the HsC8 subunit might have an important function in initiating a very early step of the ring formation.

Figure 6. HsC8-HsPROS30 rings contain at least one HsC8 subunit. a, Sepharose Q fraction 4 was depleted of HsC8 *via* three consecutive immunoprecipitation steps with anti-HsC8 coupled to Sepharose beads. The three immunoprecipitates and the final supernatant were analyzed as in Figure 5. b, The molecular mass of complexes containing only HsPROS30 (sup 3) was estimated on a Superose 6 column. Fractions were subjected to SDS-PAGE, blotted and probed with anti-HsPROS30 monoclonals.

Materials and Methods

Cloning strategies

Primers were purchased from Eurogentec. The *Nco*I and *Nde*I sites in the primer sequences are underlined and the mutations introduced in the proteasomal cDNAs double underlined.

The cloning of HsC8 cDNA has been described previously (Gerards *et al.*, 1997). The HsPROS30 (Silva-Pereira *et al.*, 1992) and HsPROS27 (Bey *et al.*, 1993) cDNAs were a gift of Dr K. Scherrer. In the HsPROS30 cDNA an *Nde*I restriction site overlapping the natural start codon was generated by site-directed mutagenesis using the oligonucleotide-directed *in vitro* mutagenesis system (Amersham) and the mutagenic primer 5'-TCTCCTGGAATCATATGCAGCTCAGC-3'. The *Nde*I-*Hinc*II fragment of the mutated cDNA and the *Hinc*II-*Bam*HI fragment (which contains a natural *Nde*I site) of the original clone were together ligated into the *Nde*I-*Bam*HI sites of the pET3c expression vector. The sequence adjacent to the natural start codon of the HsPROS27 cDNA was also converted to form an *Nde*I site by site-directed mutagenesis with the mutagenic primer: 5'-ACACACTGAACCATATG-GTGACGGG-3'. The *Nde*I-*Bam*HI fragment was cloned into the *Nde*I-*Bam*HI sites of pET3c. Mutated cDNAs were checked for correctness by sequencing with the Sequenase version 2.0 sequencing kit (United States Biochemicals).

Expression of recombinant proteins and fractionation of bacterial lysates

The proteasomal subunits were expressed separately or coexpressed with HsC8 in the *E. coli* strain BL21(DE3) (Studier *et al.*, 1990) as described previously (Gerards *et al.*, 1997). Briefly, pET-vector containing bacteria were grown in the presence of appropriate antibiotics. At an A_{550} the expression of the proteasomal subunits was induced by adding isopropyl-1-thio- β -D-galactopyranoside (IPTG; Research Organics) to a final concentration of 1 mM. Bacteria were harvested three hours after induction by centrifugation at 5000 g for 15 minutes. Bacterial pellets were resuspended in 1/25 volume of the culture volume in TEN300 (50 mM Tris-HCl (pH 8), 0.5 mM EDTA, 300 mM NaCl) and frozen at -80°C . To isolate the water-soluble fraction the bacteria were thawed at 37°C , subsequently lysed with chicken egg-white lysozyme (Sigma) and centrifuged for 30 minutes at 90,000 g at 4°C . The supernatant (water-soluble fraction) was used for isolation and analysis of the proteasomal subunits.

Purification and molecular mass determination of proteasomal subunits

Recombinant HsC8 was purified from the bacterial water-soluble fraction as described previously (Gerards *et al.*, 1997). The HsC8-HsPROS30 complexes were separated on a Sepharose Q Fast Flow column (Pharmacia) in conjunction with a Biorad Econo system. After loading the water-soluble fraction of bacteria coexpressing HsC8 and HsPROS30 the column was washed with TEN300, and proteins were eluted with a linear gradient from 300 to 600 mM NaCl. Three fractions with a different HsC8-HsPROS30 ratio were concentrated in an Amicon ultrafiltration cell with a 10 kDa cutoff filter (Filtron). For final purification and molecular mass determination the concentrated fractions were applied on a Superose 6 HR 10/30 prepacked size exclusion column (Pharmacia-LKB) on an LKB Bromma HPLC system. HsC8-HsPROS30 complexes were eluted with TEN300 at a

flow rate of 0.5 ml/minute. Absorbance was monitored at 225 nm. The column was calibrated with high molecular mass standards (Pharmacia-LKB) as indicated in the Figure legends.

Analytical gel filtration

The molecular mass of the proteasomal subunits and subunit complexes present in bacterial water-soluble fraction was estimated by FPLC gel filtration in TEN300 buffer. Three different size exclusion columns were used; Superose 6 HR 10/30, Superose 12 HR 10/30 and Superdex 75 Hiload 16/60 (Pharmacia-LKB), with separating range of 50 to 5000 kDa, 5 to 300 kDa and 5 to 70 kDa, respectively. Column fractions were analyzed by SDS-PAGE. Gels were stained with Coomassie brilliant blue or blotted on nitrocellulose and probed with antibodies directed against a specific proteasomal subunit. Monoclonal antibodies used are MCP72, directed against HsC8 (Hendil *et al.*, 1995; Kristensen *et al.*, 1994), kindly provided by Dr K. Hendil; p27k (IB5) against HsPROS27 (Grossi de Sa *et al.*, 1988) and p30-33k (62A32) against HsPROS30 (Silva-Pereira *et al.*, 1992), both kindly provided by Dr K. Scherrer or purchased from Organon Teknika. Gel filtration columns were calibrated with molecular mass markers (Pharmacia-LKB) as indicated in the Figure legends.

Immunoprecipitation

Protein A-conjugated Sepharose beads (Kem-En-Tec) were washed three times in KHN-buffer (100 mM KCl, 20 mM Hepes (pH 7.5), 0.05% NP40) and 20 μ l of beads was incubated with 30 μ l of polyclonal anti-HC8 serum in 1 ml of KHN-buffer for two hours. After three washes with KHN-buffer the beads were incubated with the Sepharose Q fractions 4, 7 and 9 for two hours. The beads were washed three times with KHN-buffer and subsequently treated with SDS-sample buffer without β -mercaptoethanol. Precipitates and supernatants were analyzed using SDS-PAGE and Coomassie brilliant blue staining of the gels.

Electron microscopy

The samples taken from the Superose 6 peak fractions containing the different HsC8-HsPROS30 complexes were diluted three times with double-distilled water (final protein concentration ± 0.1 mg/ml) and a drop was applied onto a glow-discharged, carbon-coated collodium film on a copper grid. After two minutes, excess liquid was removed using filter paper, and the sample was fixed in 1% paraformaldehyde for two minutes. Subsequently, the grid was washed with five drops of 0.1% ammonium acetate, stained with 1% uranyl acetate, and air-dried after removal of excess liquid with filter paper at the edge of the grid. The specimens were examined in a Jeol 1210 transmission electron microscope at 100 kV. Electron micrographs were recorded on AGFA Scientia EM 23D56 film at a magnification of 50,000 $\times$.

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