Dissecting the assembly pathway of the 20S proteasome

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Abstract Proteasomes reach their mature active state via a complex cascade of folding, assembly and processing events. The *Rhodococcus* proteasome offers a means to dissect the assembly pathway and to characterize intermediates; its four subunits (α_1, α_2, β_1, β_2) assemble efficiently in vitro with any combination of α and β. Assembly studies with wild-type and N-terminally truncated β-subunits in conjunction with refolding studies allowed to define the role of the propeptide which is two-fold: It supports the initial folding of the β-subunits and it promotes the maturation of the holoproteasomes.

Key words: Proteasome; *Rhodococcus*; Processing; Assembly; Propeptide

1. Introduction

The proteasome is a macromolecular assembly designed for the controlled proteolysis of either abnormal or of short-lived regulatory proteins [1]. It is found in all three urkingdoms, the archaea, bacteria and eukarya [2,3]. The 20S proteasome is built from 28 subunits, 14 of the α-type and 14 of the β-type; the overall stoichiometry is $\alpha_7\beta_7\beta_7\alpha_7$. Collectively they form a barrel-shaped complex with a diameter of 11 nm and a length of 15 nm which encloses a system of internal cavities. The central chamber and the two antechambers are connected with each other via narrow constrictions [4]. Only unfolded proteins can pass the openings which give access to the central chamber where the active sites are located [5]. Thus the degradative action becomes compartmentalized; this in turn offers means to control proteolysis. The mature active state of the 20S proteasome is reached via a folding and assembly pathway which in the case of the eukaryotic proteasome must be able to orchestrate the correct positioning of two copies each of 14 different but related subunits. Moreover, in the course of the assembly, the β-type subunits are processed by an autocatalytic mechanism [6–8] removing the propeptide and exposing the catalytic nucleophile, i.e. the N-terminal threonine [4,9].

In this communication we describe experiments aimed at dissecting the assembly pathway of the 20S proteasome and elucidating the role of the β-subunit propeptide.

2. Materials and methods

2.1. Plasmids and DNA manipulation

For cloning and expression vectors pT7-7 [10] and pRSET6a [11] were used. Construction of plasmids containing the genes of the *Rhodococcus* proteasome subunits (pT7-7-α_1, pT7-7-α_2, pT7-7-β_1 and pT7-7-β_2) was described recently [12]. Using PCR the genes encoding β_1 and β_2 were deleted for the propeptide sequence and elongated at the 3′-end by an affinity tag coding for six consecutive histidine residues (His_6). Plasmid-DNA pT7-7-β_1 and pT7-7-β_2 were used as templates. The resulting PCR products were cloned into vector pT7-7 to obtain the expression constructs pT7-7-$\beta_1\triangle$ and pT7-7-$\beta_2\triangle$. In the same way the sequence encoding the β_1-propeptide was amplified by PCR, provided with a His_6-affinity tag at its 3′-end and cloned into the high copy expression vector pRSET6a. Accuracy of all constructs was confirmed by DNA cycle sequencing (373 DNA Sequencer) using the DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). Plasmids were transformed by electroporation.

2.2. Protein expression and purification

Recombinant proteins and the β_1-propeptide were expressed in *E. coli* BL21(DE3) cells (Stratagene) and isolated by Ni-affinity chromatography (Qiagen) according to the instructions. Full length and deleted subunits were purified under native conditions as described previously [12]. The β_1-propeptide was purified under denaturating conditions (6 M urea) and refolded by stepwise dilution of urea in a dialysis procedure (for approx. 4 h in 4, 2, 1, 0.5, 0 M urea, respectively, 25 mM Tris-HCl, 1 mM EDTA, 1 mM NaN_3 pH 7.5 (TEN)). Recombinant proteins were analyzed by 16% Tricine SDS-PAGE [13] and dialyzed against TEN.

2.3. Assay for in vitro assembly

Seventy μg each of β- and α-subunits in a total volume of 250 μl TEN were incubated at 37°C and the formation of proteasomes was monitored by their ability to hydrolyze the fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC (Bachem). For this purpose 25 μl aliquots of the reaction mixture were taken at several time points and supplemented with TEN to a volume of 300 μl. Final substrate concentration was 100 μM. Proteolytic cleavage was measured continuously at 37°C on a Contron SFM 25 fluorescence spectrophotometer as described previously [12]. Rate constants for the formation of proteasomes were determined by fitting the increase of proteolytic activity to a first-order equation using the program Kaleidagraph. For native PAGE analysis (5–15% polyacrylamide gradient, Tris-Cl pH 8.8 [14]) 25 μl aliquots of the reaction mixture were mixed with loading buffer (60 mM Tris-HCl pH 8.8, 20% glycerol, 0.01% bromophenol blue), immediately frozen in liquid nitrogen and stored at −80°C. Electrophoresis was performed at 4°C and 20 mA over 4 h. For 2D gel electrophoresis bands of the nondenaturating gel were excised and applied to 16% SDS−PAGE. Protein bands were visualized by Coomassie staining. When both α-subunits were present in the reaction mixture simultaneously they were in a seven-fold molar excess with respect to the β-subunit. In experiments with exogenously added β_1-propeptide the respective β-subunit was preincubated with a molar excess of the propeptide before adding the α-subunit and starting the reaction.

2.4. Unfolding-refolding experiments

For unfolding-refolding experiments the intrinsic tyrosine fluorescence of α_1- and β_1-subunits was used as a local probe. Fluorescence spectra were measured on a Hitachi-F4500 spectrofluorimeter on excitation at 276 nm and recording the emission from 290 nm to 400 nm using 5 nm slit widths. The spectra were corrected according to sup-

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Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; AMC, 7-amino-4-methylcoumarin; Suc, succinyl; pre, precursor; pro, propeptide; βΔ, β-subunit with propeptide deleted

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pliers recommendation. In the course of unfolding the fluorescence intensity decreased slightly, the maximum at 303 nm remained constant. Proteasome subunits were unfolded in 7.0 M urea (25 mM Tris-HCl, 1 mM EDTA, pH 7.5). The refolding kinetics recorded on a stopped-flow machine (Applied Photophysics, Leatherhead, UK) were monitored by fluorescence on excitation at 276 nm using a cut-off filter of 305 nm. Refolding was initiated by 1+5 dilution in refolding buffer (25 mM Tris-HCl, 1 mM EDTA, pH 7.5) giving a final concentration of 1.2 M urea. The final concentration of α_1 and β_1^{pre} was 1.8 µM, that of $\beta_1\triangle$ 2.0 µM, and that of the propeptide 2.3 µM. Data were fitted to single or double exponential first-order kinetics using the program Kaleidagraph.

2.5. Electron microscopy and image processing

Electron microscopy and image analysis were performed as described in detail elsewhere [15]. All samples were negatively stained with uranyl acetate.

3. Results and discussion

3.1. In vitro assembly of the Rhodococcus 20S proteasome

In order to uncover the principles which govern the correct assembly of this multisubunit complex we have established an in vitro assembly system. Unlike in vivo studies where the interpretation of phenotypes is often difficult and compromised by the coexistence of wild-type and mutant subunits, the in vitro system allows to capture intermediates along the assembly pathway and to subject them to biochemical and biophysical studies.

We have chosen the 20S proteasome from the bacterium *Rhodococcus erythropolis* [16] as a model system for a number of reasons: Being built from four different subunits (α_1, α_2, β_1, β_2) it is of tractable complexity. Previous coexpression studies had shown that both α-type subunits can interact productively with both β-type subunits [12]; thus it is possible to assemble functional proteasomes from any combination of α- and β-subunits. The four subunits can be expressed separately, the β-type subunits with and without their prosequences, and the propeptides separately; individually all subunits remain monomeric and inactive, unlike the *Thermoplasma* proteasome where the α-subunit assemble almost instantaneously [17]. When monomeric α- and β-subunits are allowed to interact, active proteasomes form spontaneously within minutes.

We monitored the formation of proteasomes in vitro by measuring the increase of proteolytic activity towards a synthetic fluorogenic peptide (Fig. 1A) and by native PAGE analysis (Fig. 1B). PAGE of aliquots of the reaction mixture at several time points revealed two sharp bands on the native gel: A high molecular weight band in the size range of complete proteasomes appeared after a short time-lag (t_1); its increase in intensity perfectly matched the activity profile. A second band in the molecular weight range of half proteasomes, appeared immediately after starting the reaction (t_0), but disappeared with time, indicating that it represents an assembly intermediate. By overlaying the gel with substrate solution, proteolytic activity could exclusively be attributed to the high molecular weight band (data not shown). For further characterization, both bands were excised and subjected to SDS-PAGE: As expected, the high molecular weight band contained α-subunits and mature β-subunits and thus represents holoproteasomes (Fig. 1D). In contrast, the low molecular weight band contained α-subunits and β-precursors in almost equal amounts (Fig. 1C). No mature, correctly processed β-subunits could be detected in the intermediate complexes. Obviously the cleavage of the propeptide takes place when the half proteasomes join to form the holoproteasome. On electron micrographs, holoproteasomes and half protea-

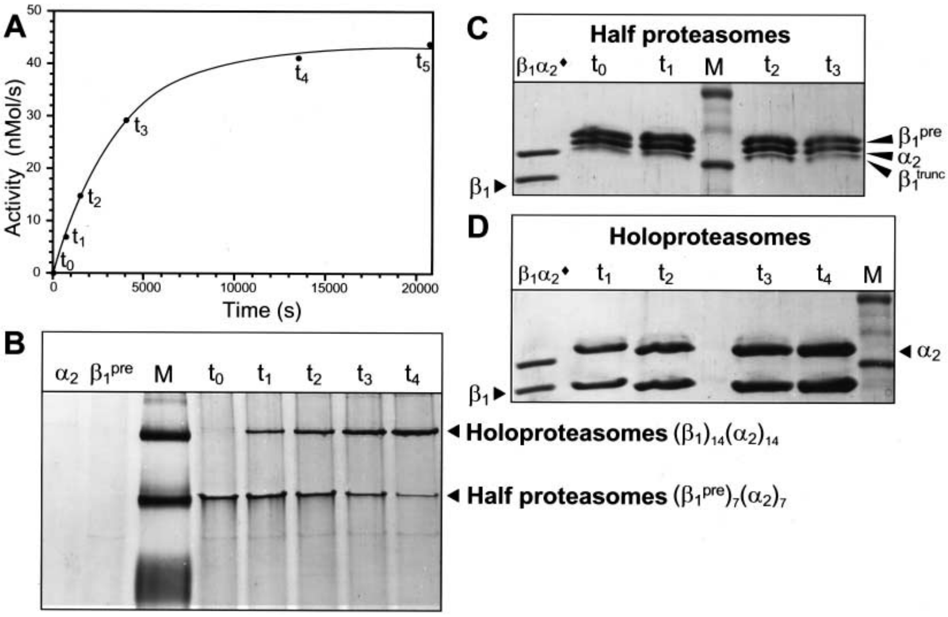

Fig. 1. Analysis of the assembly reaction in vitro. A: Suc-Leu-Leu-Val-Tyr-AMC cleaving activity of proteasomes formed during the assembly reaction. Equal amounts of β_1^{pre} (β_1-precursor) and α_2 in a total volume of 250 µl TEN were incubated at 37°C. At several time points (t_0, 30 s; t_1, 12 min; t_2, 25 min; t_3, 67 min; t_4, 225 min; t_5, 345 min) proteolytic activity was measured and analyzed as described in Section 2. B: Corresponding native PAGE analysis. Time points refer to A. As a reference the primary products (α_2 and β_1^{pre}) were loaded on the gel. M, molecular weight marker: 669, 440 and 232 kDa. C and D: SDS-PAGE analysis of the protein bands of the native gel shown in B. Bands were excised and applied to 16% Tricine SDS-PAGE. $\beta_1\alpha_2^{\blacklozenge}$, recombinant *Rhodococcus* proteasomes (α_2-subunit without His-tag, β_1-subunit with His-tag); β_1, mature β-subunits (processed); β_1^{pre}, precursor protein (unprocessed); β_1^{trunc}, truncated form of the β_1-precursor, previously identified as one of three truncated forms of the β-subunit expressed in *E. coli* (cleaved at position −48 [12]). M: 35, 31 kDa. Time points refer to A. All proteins were visualized by Coomassie blue staining.

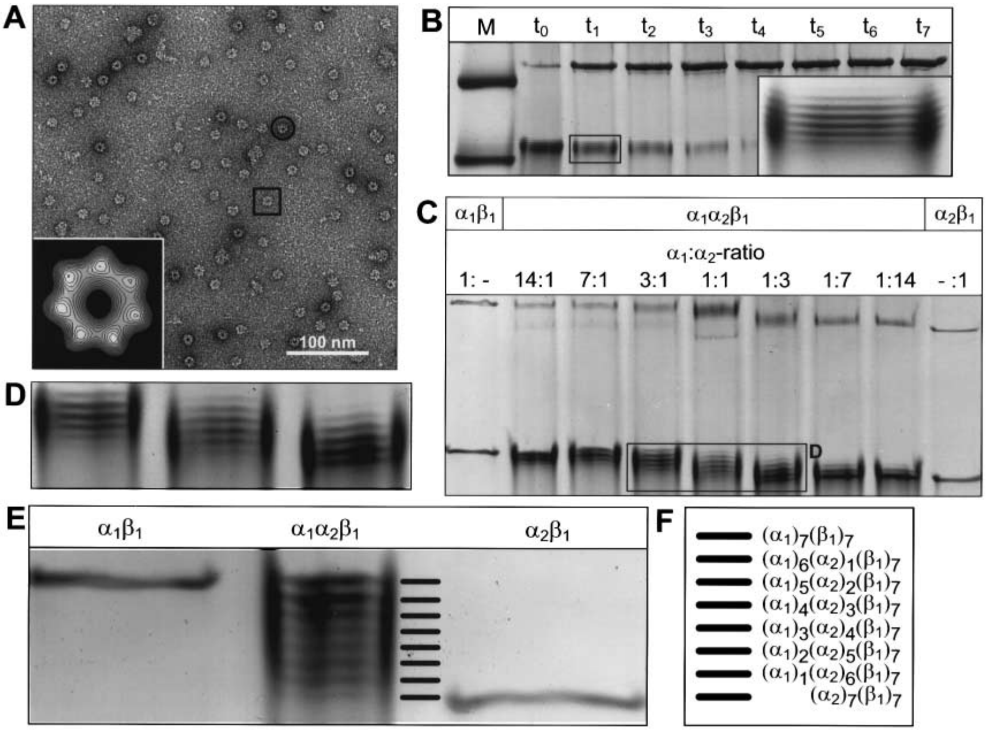

Fig. 2. Characterization of half proteasome intermediates. A: Electron micrograph of negatively stained half (rectangle) and holoproteasomes (circle) assembled from α_2- and β_1-subunits showing typical end-on views. Characteristic for holoproteasomes is the darker halo of negative stain. The inset shows an averaged projection of half proteasomes along their seven-fold symmetry axis. B: Native PAGE analysis of the in vitro asssembly at 37°C after mixing β_1^{pre}-subunits (precursors) with a seven-fold molar excess of both α_1- and α_2-subunits. Aliquots of the reaction were loaded on the gel at several time points (t_0, 30 s; t_1, 12 min; t_2, 25 min; t_3, 67 min; t_4, 225 min; t_5, 345 min). The characteristic multiplet band is marked and enlarged (inset). M: 669 and 440 kD. C: Native PAGE analysis of a series of assembly reactions after combining β_1^{pre}-subunits with different α_1/α_2-ratios and incubating for 12 min at 37°C. The respective α_1/α_2-ratio is indicated on top of every lane. D: Enlarged section of C. E: Identification of 8 distinct bands obtained with $\alpha_1\alpha_2\beta_1$ complexes. The highest and lowest bands of the multiplet represent half proteasome complexes, built up by only α_1- or only α_2-subunits, respectively. F: Table of 8 different α_1/α_2 combinations which are p213ossible for seven-membered rings.

some intermediates can be discriminated (Fig. 2A). Whereas it had been notoriously difficult to determine unambiguously the symmetry from end-on views of *Thermoplasma* or *Rhodococcus* holoproteasomes [15,16], image analysis of end-on views of half proteasomes clearly revealed their seven-fold symmetry (inset in Fig. 2A).

In order to examine whether there is any preference for specific pairs of (α_1, α_2) and (β_1, β_2), we analyzed the in vitro assembly of β_1-subunits mixed with an excess of both α-type subunits. In this experiment, the otherwise sharp band of half proteasomes on native gels transforms into a multiplet of bands (Fig. 2B). To substantiate our assumption that this multiplet splitting is due to different numbers of (larger) α_1- and (smaller) α_2-subunits in half proteasomes, we systematically varied the ratio of α_1 to α_2 and analyzed the assembly intermediates by native PAGE (Fig. 2C and D). We were able to direct the reaction from one borderline case (only α_1) to the other one (only α_2). In between, we could identify eight distinct bands (Fig. 2E), corresponding to the eight different α_1/α_2-ratios which are possible for a ring complex of seven-fold symmetry (Fig. 2F). Thus, the electrophoretic analysis corroborates the seven-fold symmetry of the complex. In addition, the distribution of intensities within the multiplet, which is close to a Gaussian distribution at equimolar ratios of α_1- to α_2-subunits, indicates that the two α-subunits interact equally efficiently with the β_1-subunits; it should be noted here that α_1 and α_2 have 81.6% sequence identity.

3.2. The β-propeptide promotes the assembly process

Like many proteases, proteasome β-subunits are synthesized as precursor proteins. In several cases it has been shown that the propeptides are transiently required for yielding an active enzyme [7,18,19]. At present, little is known about the function of propeptides of proteasome β-subunits except that their removal is required for exposing the catalytic nucleophile, the N-terminal threonine residue. The N-terminal propeptide of the yeast Doa3 β-subunit was recently reported to be essential for incorporation of this subunit into proteasome complexes [7]. In contrast, *Thermoplasma* proteasomes indistinguishable from wild-type, could be obtained with mutants of β-subunits lacking the proregion, albeit with a somewhat lower yield [17,20].

In order to define the role of the propeptides in the course of the assembly of the *Rhodococcus* proteasome, we expressed and isolated both β-type subunits without their proregions; β_1 was genetically deleted for 64 residues ($\beta_1\triangle$), β_2 for 58 residues ($\beta_2\triangle$). Both the wild-type β-subunits and the truncated proteins alone remained monomeric and proteolytically inactive, although the N-terminal threonine residue of the mutant is exposed. Coexpression of $\beta_1\triangle$ with α_1 in *E. coli* yielded active proteasomes. However, electron microscopy revealed that only approx. 20% of the purified subunits were assembled correctly indicating that the propeptide is indeed important, though not essential for formation of proteasomes in vivo. Monitoring proteasome formation in vitro by proteolytic activity as a global probe clearly demonstrated the importance of the β-propeptide. The increase of activity was drastically slowed down in the absence of the β-propeptide (Fig. 3A), and native PAGE analysis confirmed the retarded formation of half proteasome intermediates and of holoproteasome complexes (Fig. 3B/C). It is noteworthy, that these intermediates in spite of the removal of the β-propeptide remain proteolytically inactive (data not shown). Thus, removal of the β-propeptide is not sufficient for activation of the enzyme. Additional interactions between β-subunits across the interface of the two central β-rings within the holoproteasome are required for active site formation as was recently proposed by Chen and Hochstrasser [7].

3.3. *The β-propeptide functions in trans*

In order to define the role of the propeptide more precisely, we expressed and isolated the β_1-propeptide. Function of a propeptide in trans, i.e. intermolecularly, would not be without precedent. For several bacterial proteases and for the proteasomal β-subunit Doa3 from yeast it was reported that direct covalent linkage of pro and protease domain is not required for yielding an active enzyme [7,21–23]. We therefore added the 72 residue β_1-propeptide (pro) exogenously to the assembly mixture of $\beta_1\Delta$ and α_1. The activity profile obtained in this experiment (Fig. 3A) showed a rapid increase of activity at early stages of the reaction. Varying the amount of exogenously added propeptide confirmed that the propeptide is most efficient at a molar ratio of 1:1; a molar excess of propeptide does not further accelerate the reaction. Thus, being a single turnover catalyst it fits perfectly into the concept of intramolecular chaperones [19]. Corresponding native PAGE analysis (Fig. 3D) demonstrated how the generation of holoproteasomes is promoted by the separately added propeptide. Interestingly, half proteasomes were not observed in this experiment, not even at $t_0 < 1$ min. Only when the amount of propeptide was significantly reduced, half proteasomes were detectable (data not shown). This implies that they have a short life-time when sufficient amount of propeptide is available and that the autocatalytic removal of the propeptide is a rate limiting step in the assembly. It is noteworthy that in spite of the divergence of the β_1- and β_2-propeptides the propeptide of the β_1-subunit supports the assembly of β_2 with equal efficiency.

The increase of proteolytic activity as a result of the three different assembly reactions ($\alpha_1+\beta_1^{pre}$ (1), α_1+$\beta_1\Delta$ (2) and α_1+$\beta_1\Delta$+pro (3)) were fitted to first-order kinetics (Fig. 3A); rate constants and amplitudes (yield of proteolytic activity) are summarized in Table 1. The progress curve of assembly reaction 3, where the propeptide was added exogenously was obtained by a double exponential first-order fit. It shows

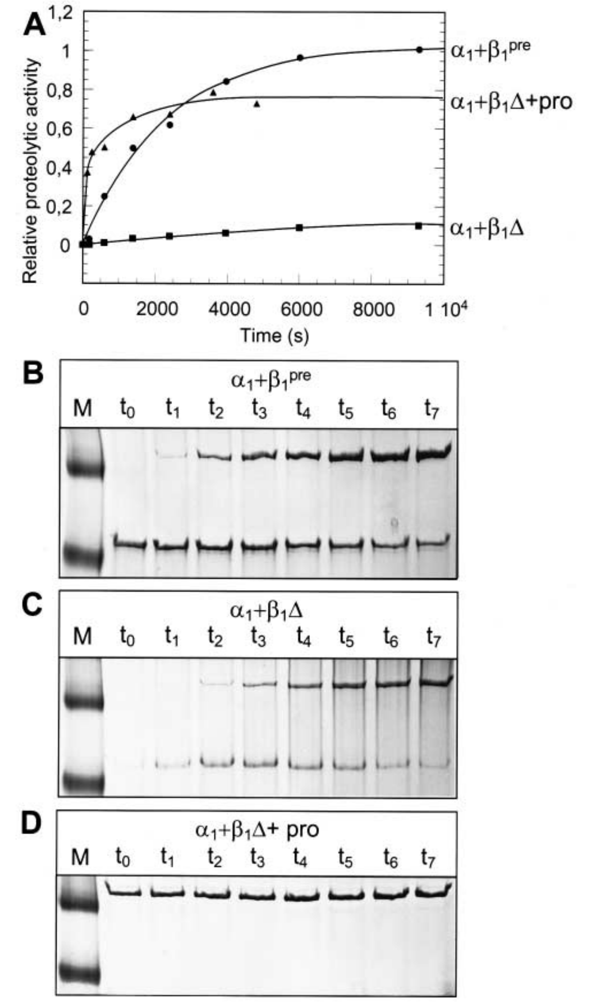

Fig. 3. Proteasome assembly monitored by proteolytic activity as a global probe and corresponding native PAGE analysis. A: Time-dependent increase in Suc-Leu-Leu-Val-Tyr-AMC cleaving activity for three in vitro assembly reactions using α_1-subunits and (1) wild-type β_1-subunits ($\alpha_1+\beta_1^{pre}$), (2) propeptide-deleted β_1-subunits (α_1+$\beta_1\Delta$) and (3) propeptide-deleted β_1-subunits supplemented with exogenously added propeptide (α_1+$\beta_1\Delta$+pro). Data of assembly reactions (1) and (2) were fitted to single exponential first-order equations, data of reaction (3) to a double exponential first-order equation. Reactions were performed as described in Section 2. A molar excess of propeptide was preincubated with $\beta_1\Delta$ before adding the α-subunit to start the reaction. B–D: Corresponding native PAGE analysis of the three reactions described in A. Time points: t_0, 30 s; t_1, 3 min; t_2, 10 min; t_3, 23 min; t_4, 40 min; t_5, 66 min; t_6, 100 min; t_7, 155 min. M: 669, 440 kDa. Equal amounts of protein were used in all reactions.

Table 1
Rate constants for the formation of mature proteasomes

Assembly reaction	Proteins	k (s^{-1})	Yield of proteolytic activity (%)
1	$\alpha_1+\beta_1^{pre}$	4.3×10^{-4}	100
2	α_1+$\beta_1\Delta$	2.1×10^{-4}	12
3	α_1+$\beta_1\Delta$+propeptide	$0.2^{a}/9\times10^{-4}$	$41^{a}/36$ 77^{b}

Rate constants of proteasome formation were derived from a fit of the increase in proteolytic activity of the formed proteasomes according to a first-order reaction. Test as described in Section 2.

[a] Rate constant and yield of the first burst phase.

[b] Sum of the yield of the first and the second phase.

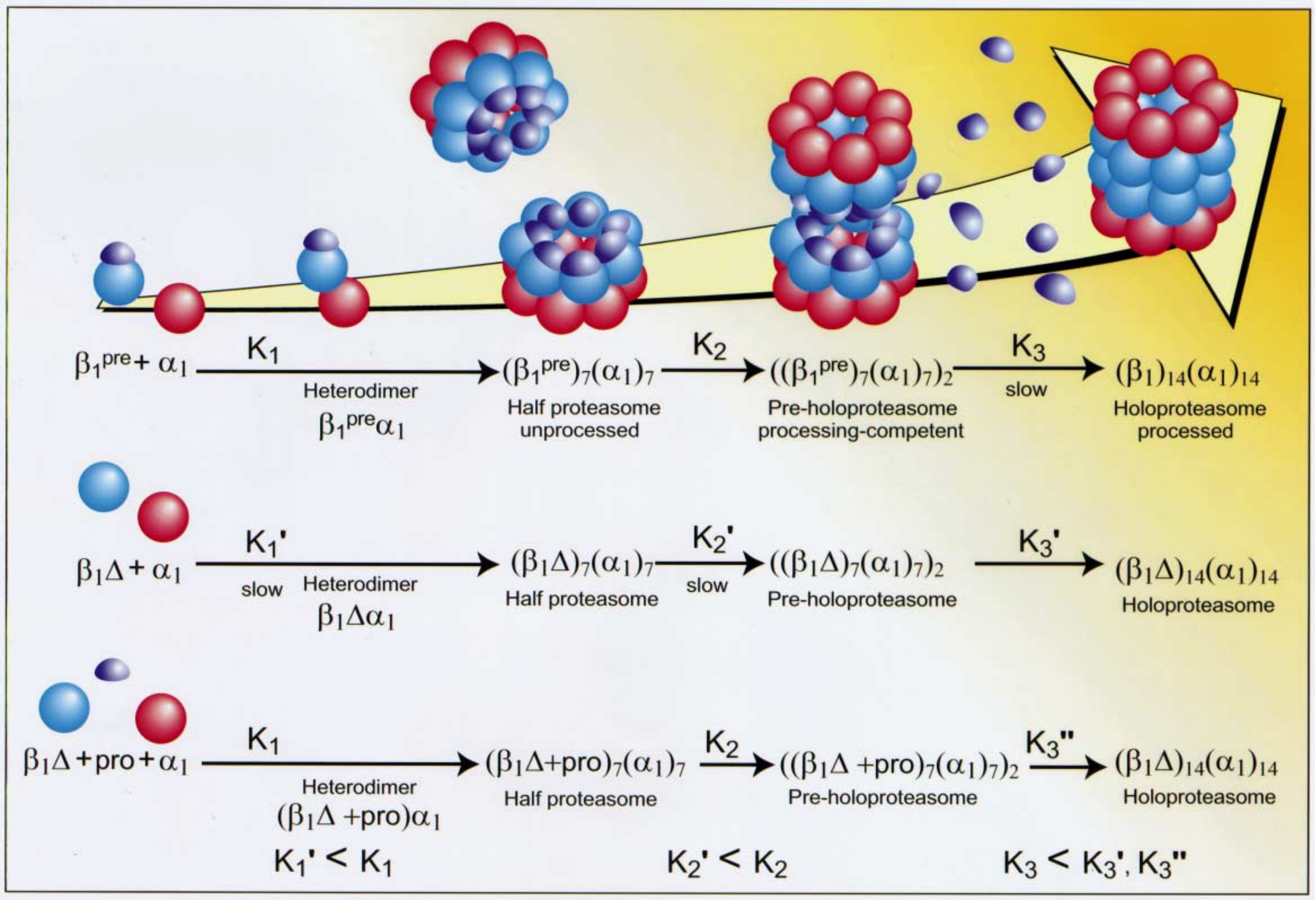

Fig. 4. Proposed model for the assembly pathway of the *Rhodococcus* proteasome.

an initial burst phase with a rate constant which is three orders of magnitude higher than the rate constants of the following phase or those derived from assembly reaction 2 (propeptide deleted) or 1 (wild-type), respectively. Such a burst phase is also reflected by the rapid appearance of holoproteasomes demonstrated on the corresponding native gel (Fig. 3D). The final yield of proteolytic activity resulting from the assembly of wild-type subunits is not nearly reached in the absence of the propeptide (12%) and also not completely, if propeptide is added exogenously (77%) demonstrating the importance of the propeptide for the maturation of the functional enzyme.

Table 2
Rate constants for refolding of proteasome subunits

Proteins	k_1 (faster phase) (s^{-1})	k_2 (slower phase) (s^{-1})
α_1	n.d.	0.026
β_1^{pre}	17.8	0.295
$\beta_1\Delta$+propeptide	n.d.	0.484

Proteasome subunits were unfolded in 7.0 M urea and refolding was initiated by 1+5 dilution in refolding buffer giving a final concentration of 1.2 M urea. The refolding kinetics recorded on a stopped-flow machine were monitored by fluorescence on excitation at 276 nm using a cut-off filter of 305 nm. Data were fitted to single or double exponential first-order kinetics.

n.d., fast folding phase observed but not exactly determined under the conditions used.

3.4. Folding of β-subunits requires the propeptide

Additional information about the kinetics of proteasome formation was obtained by unfolding-refolding experiments of proteasomal subunits applying stopped-flow techniques. The intrinsic tyrosine fluorescence of α_1- and β_1-subunits was used as local probe. The progress curves show two phases both for α_1, β_1 and $\beta_1\Delta$ plus propeptide. The data were fitted to double exponential first-order kinetics and are summarized in Table 2. The progress curve which resulted from $\beta_1\Delta$ without propeptide could not be attributed to proper refolding. We conclude that the propeptide is required for the refolding of β-subunits.

3.5. A model for the assembly pathway

Taken together our results allow to put forward a model for the assembly pathway of the eubacterial proteasome (Fig. 4); the key features of this model are likely to apply also to eukaryotic proteasomes. The time needed for folding of the isolated subunits is much shorter than for the subsequent association reactions (Tables 1 and 2). The final state of proteasome assembly was detected by proteolytic activity as a global probe, the existence of enzymatically inactive half proteasomes could be demonstrated by native PAGE and electron microscopy. The existence of α/β heterodimers is not formally proven yet but given the fact that neither α- nor β-subunits alone yield any detectable assembly product (Fig. 1B), it is likely to exist. Obviously, we can not measure rate constants for this initial step of assembly. Assuming an association step of half proteasomes as rate limiting step in holoproteasome formation, the reaction is expected to be bimolecular. However, the reaction profile obtained by assaying the increase in enzymatic activity could not be fitted to a bimolecular reaction; formally a monomolecular reaction (Fig. 3A) according to the scheme:

$$2\mathrm{A} \xrightarrow{k_a} \mathrm{B} \xrightarrow{k_b} C$$

is observed, where A stands for half proteasome, B for pre-holoproteasome and C for mature holoproteasome. The experimental data reveal the bimolecular rate constant k_a not to be rate limiting; the association of unprocessed half proteasomes as a bimolecular reaction must be faster than the following monomolecular reaction (k_b). We relate this last reaction to the processing of the pre-holoproteasome i.e. the cleavage of the propeptide and a concomitant conformational change. The β-propeptide is critical for both, initial folding of β-subunits and final maturation of holoproteasomes. Its role in efficient folding of β-subunits is reflected by the significant retardation of half proteasome formation observed with the assembly reaction of $\beta_1\Delta+\alpha$. Whereas in this reaction half proteasome intermediates were detectable, addition of propeptide reduces their life-time dramatically. This fact is clear evidence for the involvement of the β-propeptide in the final step of proteasome maturation.

In eukaryotic proteasomes the propeptides of the β-type subunits are extremely divergent and vary in length from 4 to 72 residues. It appears unlikely that they are equally efficient in supporting the folding and maturation of their respective subunits. In fact the seven residue propeptide of the *Thermoplasma* β-subunit has no significant effect on the kinetics of the assembly of the *Rhodococcus* proteasome (data not shown). It appears conceivable that different folding efficiencies of the propeptides of eukaryotic proteasomal subunits generate a timing pattern which determines the sequence of subunit incorporation into the complex and thus help to orchestrate the assembly.

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