Mass Spectrometry Reveals the Missing Links in the Assembly Pathway of the Bacterial 20 S Proteasome**

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The 20 S proteasome is an essential proteolytic particle, responsible for degrading short-lived and abnormal intracellular proteins. The 700-kDa assembly is comprised of 14 α-type and 14 β-type subunits, which form a cylindrical architecture composed of four stacked heptameric rings (α-β-α-β-α-β-α-β). The formation of the 20 S proteasome is a complex process that involves a cascade of folding, assembly, and processing events. To date, the understanding of the assembly pathway is incomplete due to the experimental challenges of capturing short-lived intermediates. In this study, we have applied a real-time mass spectrometry approach to capture transient species along the assembly pathway of the 20 S proteasome from Rhodococcus erythropolis. In the course of assembly, we observed formation of an early α/β-heterodimer as well as an unprocessed half-proteasome particle. Formation of mature holoproteasomes occurred in concert with the disappearance of half-proteasomes. We also analyzed the β-subunits before and during assembly and reveal that those with longer propeptides are incorporated into half- and full proteasomes more rapidly than those that are heavily truncated. To characterize the preholoproteasome, formed by docking of two unprocessed half-proteasomes and not observed during assembly of wild type subunits, we trapped this intermediate using a β-subunit mutational variant. In summary, this study provides evidence for transient intermediates in the assembly pathway and reveals detailed insight into the cleavage sites of the propeptide.

The 20 S proteasome is a macromolecular assembly designed for the controlled proteolysis of short-lived regulatory proteins as well as abnormal and misfolded proteins (1, 2). The structure of this 700-kDa particle is highly conserved and can be found in eukaryotes, archaebacteria, and some eubacteria (3). It is composed of 28 subunits, arranged in a cylindrical architecture consisting of four heptameric rings: two outer α-type subunit rings embracing two central β-type subunit rings (α-β-α-β-α-β). The β-subunits contain the active proteolytic sites. The main difference between the prokaryotic and eukaryotic 20 S proteasomes is that of complexity. Prokaryotic 20 S proteasomes (e.g. from Thermoplasma acidophilum (4, 5)) are generally composed of identical copies of 14 α-subunits and 14 β-subunits; eukaryotic proteasomes recruit α- and β-subunits from 14 different subfamilies (6, 7). In mammals, complexity is enhanced further by three γ-interferon inducible subunits, which are incorporated into the proteasome during the immune response (for a review, see Ref. 8). The bacterial proteasome from the actinomycetes Rhodococcus erythropolis is intermediate in complexity, consisting of two α-type and two β-type subunits. Any of the four possible combinations of α- and β-type subunits assembles to fully active proteasomes in vivo and in vitro (9).

β-subunits of 20 S proteasomes from both eukaryotes and prokaryotes are expressed in a precursor form, with a propeptide sequence at the N terminus. Interestingly, the propeptides from different organisms are highly divergent and are thought to play different roles in proteasome assembly and activation (10, 11). During the course of assembly, they are post-translationally processed and removed (12). This autocatalytic mechanism exposes the catalytic nucleophile (i.e. the N-terminal threonine) and ensures that active sites are formed only after completion of the assembly process (13). In contrast to the short and often dispensable propeptides of the archaenal β-type subunits (14–16), the Rhodococcus β1- and β2-subunits are translated as precursor proteins with relatively long propeptides of 65 and 59 residues, respectively. In the absence of these propeptides, the rate of formation of the proteasome complex is retarded (13). In general, the propeptide sequence of eubacteria comprises a region of about 15 amino acids near the center of the propeptide (Ser-42 to Pro-27), which exhibits significant conservation (17). Designated the “central box,” this region is assumed to be important for propeptide function. Similarly, propeptides from eukaryotic β-type subunits comprise more than 70 residues and have been shown to be essential for proteasome biogenesis and cell viability (18–20).

The relatively low complexity of archaenal 20 S proteasomes has allowed the first studies of assembly. When archaenal proteasomal α- and β-subunits are co-expressed in Escherichia coli, mature and fully active 20 S proteasomes are formed (16, 21). In vitro studies of subunits individually produced demonstrate that the archaenal assembly appears to be conserved (12, 14, 15, 22) with the formation of seven-membered α-subunit rings providing a platform or template onto which the β-sub-
monitor the formation of an early assembly intermediate, namely the \( \alpha/\beta \)-heterodimer, the half-proteasomes, as well as mature 28-subunit 20 S proteasomes. In addition, we were able to determine in great detail the sites that are cleaved within the \( \beta \)-subunit proteptides during the different assembly states. More generally, besides providing valuable insight into the assembly process, the results highlight the power of this method for structural biology and biogenesis studies of macromolecular complexes in particular.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Mutagenesis**—For reasons of simplicity and the availability of the crystal structure of a simplified \( \alpha1\beta1 \) 20 S proteasome from *R. erythropolis* (24), only one type of \( \alpha \)-subunit and one type of \( \beta \)-subunit was used for the *in vitro* and *in vivo* assembly experiments. The single gene constructs pT7-7 \( \alpha1\text{His}_{16} \) and pT7-7 \( \beta1\text{His}_{16} \) encoding the C-terminal His-tagged \( \alpha \)- and \( \beta \)-subunit, respectively, were used for the *in vitro* assembly experiments. For the *in vivo* assays, the bicistronic expression plasmid (pT7-7 \( \beta1\text{His}_{16}-\alpha1 \)) was used for recombinant co-expression of the genes encoding the \( \alpha1 \) and the \( \beta1 \)-subunit (tagged with a C-terminal His tag) leading to active, mature proteasomes assembled *in vivo*. All plasmids were a kind gift of Dr. Frank Zühl.

The latter plasmid was also used as a template for site-directed mutagenesis experiments. The \( \beta1\text{His}_{16} \)-subunit residues Asp173 and Asp176 were mutated to alanines in the single gene construct using the QuickChange site-directed mutagenesis kit from Stratagene. The experiments were performed according to the manufacturer’s manual. The sequence of the mutant construct was verified by non-radioactive DNA sequencing.

**Expression and Purification**—Cells of the *E. coli* strain BL21 (DE3) (Stratagene) were transformed with the respective expression plasmid and grown in 6 liters LB medium to mid-log phase at 30 °C. After induction with a final concentration of 1 mM isopropyl-\( \beta \)-D-thiogalactopyranoside for 5 h, cells were harvested and resuspended in sonication buffer (20 mM sodium phosphate, 50 mM NaCl, pH 7.4) containing a protease inhibitor mixture (Complete EDTA-free (Roche Applied Science) used according to the manufacturer’s specifications), treated for 30 min on ice with lysozyme 1 mg/ml (Sigma) and a few grains of DNase I (Roche Applied Science), and sonicated for 15 min (Sonifier 250, Branson). The lysate was further fractionated first by a low speed spin (6000 \( \times \) g, 15 min) followed by a high-speed spin (30,000 \( \times \) g, 30 min). The supernatant was filtered and directly loaded onto a 1-mL His-trap column (GE Healthcare), previously equilibrated with buffer A (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4). Protein bound nonspecifically was removed with 10 column volumes of buffer A. Assembled proteasomes as well as single subunits were eluted with a gradient of 20–500 mM imidazole. Fractions were analyzed by 12% Schaegger SDS-PAGE and 4–20% Novex Tris-glycine native PAGE (Invitrogen). Fractions containing the designated proteins were pooled, concentrated, dialyzed against gel filtration buffer (20 mM Hepes, 150 mM NaCl, pH 7.5), and applied to a High Load 16/60 Superdex 200 (GE Healthcare) previously equilibrated with gel filtration buffer. Fractions were

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7 The abbreviations used are: MS, mass spectrometry; Suc, succinyl; AMC, amido-4-methylcoumarin.
analyzed by 12% Schaegger SDS-PAGE and 4–20% Novex Tris-glycine native PAGE (Invitrogen). Those fractions containing 20 S proteasomes or the individual subunits were pooled, concentrated, and used for subsequent analysis.

Native PAGE Analysis—4–20% Novex Tris-glycine native PAGE (Invitrogen) was used to probe the assembly state of in vitro assembled α- and β-subunits. 1 nM of each individually purified subunit was mixed in a final total volume of 250 μl of the assay buffer (20 mM HEPES, 150 mM NaCl, pH 7.5). The reaction mixture was incubated at 37 °C for 30 s, 2 min, 5 min, 30 min, 1 h, 2 h, or overnight. Reactions were quenched by flash-freezing the samples in liquid nitrogen, and the samples were applied to the native PAGE run at 4 °C. The in vivo assembled wild type proteasome and the individual purified subunits were used as controls.

Proteolysis Assay—The synthetic fluorogenic peptide Suc-LLVY-AMC (Bachem, Heidelberg, Germany), dissolved in Me₂SO, was used as a substrate for measuring the proteolytic activity (13). Fluorescence intensity was measured by a FluoStar Optima spectrophotometer at a reaction temperature of 37 °C ± 0.5 °C. Either 200 pmol of β-subunit or 14.3 pmol of in vivo assembled holoproteasome was equilibrated with assay buffer (20 mM HEPES, 150 mM NaCl, pH 7.5) in 96-well microtiter plates at 37 °C. After about 10 min, 50 μl of 40 μM substrate solution (0.5% final concentration Me₂SO) was added to the assay solution containing the individual β-subunit and incubated for a further 10 min. The reaction was initiated by the addition of either 200 pmol of α-subunit to the β-subunit/substrate solution or 50 μl of 40 μM substrate solution (0.5% final concentration of Me₂SO) to the holoproteasome, resulting in equimolar amounts of either in vitro or in vivo assembled proteasomes and a final concentration of 10 μM Suc-LLVY-AMC in a final total volume of 200 μl. Fluorescence of the reaction mixture was assayed immediately. Reaction progress was monitored at 53-s intervals by measuring the relative fluorescence of each well using excitation and emission wavelengths of 320 and 460 nm, respectively. Each experiment was repeated at least three times.

Mass Spectrometry—Nanoflow electrospray ionization (ESI)-MS and tandem MS experiments were conducted on a high mass Q-TOF type instrument (30) adapted for a QSTAR XL platform (31). Conditions were carefully chosen to allow the ionization and detection of proteasome assemblies without disrupting non-covalent interactions. Prior to MS analysis, 0.3–0.6 mg/ml aliquots of α- and β-subunits were concentrated 5–10 times and buffer-exchanged into 1 M ammonium acetate solution using Nanosep (Pall) columns. Subsequently, the concentrations of the α- and β-subunit-containing solutions were determined by UV absorbance. The assembly reaction was analyzed at 4, 25, and 37 °C after mixing equimolar quantities of the α- and β-subunits at various time intervals until the reaction was complete. The first time point was taken 2 min after incubating the two subunits.

Typically, aliquots of 2 μl of solution were electrospayed from gold-coated borosilicate capillaries prepared in-house as described (32). The following experimental parameters were used: capillary voltage up to 1.2 kV, declustering potential 100–150 V, focusing potential 200–250 V, declustering potential between 30 s and 16 h after introducing the subunits. 

**FIGURE 2. Biochemical analysis of the in vitro assembly process.** A, Coomassie Blue-stained native PAGE of the time-dependence of in vitro assembly. Equimolar quantities of individually purified wild type α1- and β1-subunits were incubated at 37 °C for 30 s (lane 1), 2 min (lane 2), 5 min (lane 3), 30 min (lane 4), 1 h (lane 5), 2 h (lane 6), or overnight (lane 7) and applied to PAGE analysis. The “high molecular weight calibration kit” for native electrophoresis (Amersham Biosciences) was used: tyrosolubulin (669 kDa), ferreroxidase (440 kDa), catalase (232 kDa), aldolase (140 kDa), and bovine serum albumin (67 kDa). B, proteolytic activity occurring during the in vitro assembly process is assayed by the time-dependent relative increase in fluorescence caused by the release of the fluorescent dye during hydrolysis of the Suc-Leu-Leu-Val-Tyr-AMC. The reaction progress curve is referred to as α1+β1 in vitro. As a control, the reaction progress curve for the proteolytic activity of the in vivo assembled proteasome, referred to as (α1β1) in vivo, was probed under the same conditions.

RESULTS

Biochemical Analysis of the in Vitro Assembly—The formation of the fully assembled proteasome complex was monitored initially in a time-dependent manner by native PAGE analysis (Fig. 2A). The individual α1- and β1-subunits give rise to bands corresponding to trimeric α1-subunits and individual β1-subunits, which appear as more than one distinct band, indicating some proteolysis. Assembly was probed at several time points between 30 s and 16 h after introducing the α1- and β1-subunits at 37 °C (Fig. 2A). Two bands appear initially, one in the
molecular mass range of a half-proteasome (~440 kDa). The second band has a higher molecular mass (>669 kDa) and corresponds to the holoproteasome (α₂β₂β₂α₂). Despite the fact that aliquots of the assembly assay were flash-frozen, the only detectable intermediate by native PAGE is the half-proteasome. The appearance of fully assembled proteasomes is in accord with the disappearance of half-proteasomes, and depletion of the individual subunits is in agreement with previous results (13).

To compare the activity of proteasomes assembled in this in vitro assembly reaction with those isolated after assembly in vivo, we used a fluorescence-based assay. We compared the rate of proteolysis of a fluorogenic peptide substrate during in vitro assembly with that of active, mature proteasomes assembled in vivo. Prior to cleavage of the peptide substrate, fluorescence is quenched, whereas upon proteolytic cleavage, an increase in fluorescence can be monitored. For in vivo assembled proteasomes, this proteolysis assay is characterized by a steep initial increase in fluorescence. The slope can be fitted to a first order kinetic whose saturation (at ~250 s) is due to substrate depletion (Fig. 2B). This demonstrates that there is no lag phase in the cleavage reaction under these conditions. Rather, cleavage is initiated immediately after the in vivo assembled proteasome is introduced to the peptide. By contrast, for the in vitro assembly reaction, in which individual α-subunits are introduced to β-subunits together with the fluorogenic substrate, an initial lag phase is clearly observed. Moreover, activity is found to increase five times more slowly than for the corresponding reaction with in vivo assembled proteasomes. This shows that despite the assembly reaction producing high molecular weight species after ~120 s (Fig. 2A), activity is gained at a much slower rate, with saturation occurring after ~1400 s. This difference between assembly rate and activity can be explained by the requirement for processing of β-subunits before full proteolytic function of the proteasome is achieved in vitro.

**Monitoring the in Vitro Assembly by MS**—Nanoflow ESI mass spectra were recorded for the individual subunits of the proteasome, the α₁- and β₁-subunits, prior to carrying out the assembly reaction, Fig. 3. For the α₁-subunit, only one major series of peaks is observed (Fig. 3A), with a measured mass consistent with that calculated for an α₁-subunit without the first Met residue and including a C-terminal His₆ (Table 1). Additional series of peaks are assigned to minor populations of α₁/α₁-homodimers and trimers, emphasizing the tendency of the α₁-subunit to aggregate. This observation is in agreement with the native PAGE analysis of α₁-subunits. In the spectrum recorded for the β₁-subunits (Fig. 3B), we could not identify full-length β₁-subunits (31,983 Da including the His₆ tag). However, a set of 12 degradation products was assigned, corresponding to removal of the first 18, 22, 23, 25, 28, 29, 32, 36, 40, 42, 48, and 50 residues of the propeptide, denoted Δ₁₈, Δ₂₂, etc. (Table 1). The predominant degradation product is the Δ₁₈ species. From the masses of these species, we can determine that all the cleavage sites in these degradation products are located within the propeptide sequence, residues Ser-48 to Thr-16 (Table 1, Fig. 3E).

**FIGURE 3.** Electrospray MS analysis of the monomeric α₁- and β₁-subunits during the assembly reaction. A–C, mass spectra of the individual α₁- and β₁-subunits prior to assembly and 12 min after initiation of the assembly reaction at 37 °C (C). D, β₁-subunits dissociated from the half-proteasome during tandem MS 16 h after the start of the assembly reaction at 25 °C. E, Propeptide sequence of the β₁-subunit from the Rhodococcus 20 S proteasome. The central box region is highlighted in yellow (E).
Monitoring the Assembly of the 20 S Proteasome

Table 1

| Protein complex     | Theoretical mass (Da) | Experimental mass (Da) | Figure No. |
|---------------------|-----------------------|------------------------|------------|
| α                   | 29,004±a              | 29,005±2               | 3, A and D |
| β (pro)             | 31,983±a              |                        |            |
| β Δ18               | 29,969                | 29,969±3               | 3, B and D |
| β Δ22               | 29,563                | 29,563±4               | 3B         |
| β Δ23               | 29,451                | 29,454±4               | 3B         |
| β Δ25               | 29,276                | 29,278±2               | 3, B–D     |
| β Δ28               | 28,913                | 28,914±2               | 3, B–D     |
| β Δ29               | 28,749                | 28,751±2               | 3, B–D     |
| β Δ32               | 28,423                | 28,425±2               | 3, B–D     |
| β Δ36               | 27,989                | 27,989±1               | 3, B and D |
| β Δ40               | 27,537                | 27,538±4               | 3, B–D     |
| β Δ42               | 27,266                | 27,269±3               | 3, B–D     |
| β Δ48               | 26,578                | 26,581±4               | 3, B and D |
| β Δ50               | 26,390                | 26,391±3               | 3, B and D |
| β (mature)          | 24,953                |                        |            |
| α/α′–homodimer      | 58,008                | 58,019±4               | 5          |
| α/β(Δ25)–heterodimer| 58,280                | 58,290±14              | 5          |
| α/β(Δ28)–heterodimer| 57,917                | 57,929±13              | 5          |
| α/β(Δ29)–heterodimer| 57,753                | 57,734±15              | 5          |
| α/β(Δ32)–heterodimer| 57,427                | 57,443±18              | 5          |
| α/β(Δ34)–heterodimer| 56,541                | 56,546±26              | 5          |
| α,b (pro)           | 426,909               | 433,044±44             | 4, A and B |
| α1,β(b(mature))     | 755,398               | 757,493±123            | 4, A and B |
| α1,β(pro)11,β(Δ18)  | 810,869               | 811,053±75             | 6D         |
| α1,β(pro)12,β(Δ18)  | 808,855               | 809,090±76             | 6D         |
| α1,β(pro)13,β(Δ18)  | 806,841               | 807,026±66             | 6D         |
| α1,β(pro)14,β(Δ18)  | 804,827               | 805,445±74             | 6D         |
| α1,β(pro)15,β(Δ18)  | 782,688               | 782,906±63             | 6E         |
| α1,β(pro)16,β(Δ18)  | 780,674               | 780,933±121            | 6E         |
| α1,β(pro)17,β(Δ18)  | 778,660               | 778,865±64             | 6E         |
| α1,β(pro)18,β(Δ18)  | 776,646               | 777,175±105            | 6E         |

a The sequence includes a C-terminal His tag.
b The initiator Met is removed.
c In vivo assembly of the β-subunit D173A,D176A mutant including a His6 tag.

To examine the assembly pathway in detail, we used MS, initially monitoring the reaction at 37 °C to enable comparison with results obtained by native gel analysis and the activity assay. Mass spectra were acquired at different time intervals after incubating equimolar ratios of the α1- and β1-subunits. 12 min after initiating the reaction, a charge state series corresponding to an unprocessed half-proteasome is observed (Fig. 4A and Table 1). Subsequently, after 25 min, we observed spectra in which both half-proteasomes and intact holoproteasomes were present, demonstrating the co-existence of both species in solution. After 29 min, half-proteasomes could no longer be detected, only holoproteasomes, in accord with the data from the native gel analysis (Fig. 2A). Since no further changes in mass spectra were observed after this time, we conclude that the assembly reaction is complete. Interestingly, although the reaction was monitored at various time points until completion, we could not identify the predicted preholoproteasome species (13). We therefore repeated the same experiments at lower temperatures, 25 and 4 °C, in an attempt to capture this intermediate and to trap populations of the half-proteasome. However, although at 25 °C we were able to trap half-proteasomes for further study, preholoproteasomes were not detected. Also, to our surprise, at these temperatures (4 and 25 °C), assembly was not complete. Even after 5 days, unprocessed half-proteasome particles persist at 25 °C (Fig. 4B). This observation indicates that the assembly processes is temperature-dependent.

If we now turn our attention to the low m/z region of the spectra recorded during assembly, we can monitor the proportions of the α1- and β1-subunits that remain in solution during the progression of the reaction. Simultaneously, with the formation of unprocessed half-proteasome and holoproteasome, the intensity of the monomeric subunits was found to decrease. A set of seven β1-subunits remained in solution 12 min after the assembly reaction was initiated at 37 °C (Fig. 3C). This subset of seven corresponds to those peptides with the shortest propeptide (from Δ25 to Δ42), implying that the β1-subunits with the longest propeptides identified prior to assembly (Fig. 3B), namely Δ18, Δ22, and Δ23, are either promptly integrated within the assembling proteasome or rapidly degraded. Both α1-subunits and truncated β1-subunits could still be detected even after the assembly reaction was complete. We attribute this observation to properties of the β1-subunits. Specifically, we anticipate that extensive truncations may perturb the native state of the β1-subunits, and consequently, preclude their successful incorporation into the holoproteasome.

To confirm the incorporation of β-subunits with the longest propeptides within the assembled form of the half-proteasome, ions of this complex at m/z 9000 were isolated and subjected to a tandem MS experiment. This process results in the expulsion of individual β-subunits and the formation of a “stripped” half-proteasome complex (α1,βn) (25). Surprisingly, a set of 10 β-subunits was identified and cleaved within the propeptide region to different extents (Δ18, Δ25, Δ28, Δ29, Δ32, Δ36, Δ40, Δ42, Δ48, and Δ50) (Table 1 and Fig. 3D). This group of β-subunits contains a range of truncated forms from Δ18 to Δ50 and is very similar to the set of monomeric β1-subunits identified prior to assembly (Fig. 3B). The major difference is in the intensity ratio of the individual species. Specifically, prior to assembly, the Δ25 species was predominant, whereas after assembly, the Δ50 species gave the highest intensity peaks. Consequently, we have found that the β-subunits expelled from the half-proteasome have undergone more extensive processing to form shorter propeptide sequences than the β-subunits prior to assembly.

A close inspection of the mass spectra recorded while monitoring the assembly, in the range of 3000–3700 m/z at all three temperatures (4, 25, and 37 °C) (Table 1 and Fig. 5), identified the appearance of two additional species. The predominant species is consistent with the α1/α′-heterodimers, emphasizing the tendency of the α-subunit to aggregate as observed in the native gel and mass spectra (Figs. 2A and 3A). Interestingly, all other species correspond to α/β-heterodimers in which the first 25, 28, 29, 32, and 40 residues of the β1-subunit propeptide are truncated. This set of α/β-heterodimers corresponds closely to the monomeric β1-subunits that were not incorporated during the assembly of the half-proteasome and holoproteasome (Fig. 3C). A possible explanation for this observation is that the reduced activity of these β1-subunits results in their becoming trapped within the α/β-heterodimers, which are not able to assemble into the full proteasome.
Mass Spectrometry Analysis of the in Vivo Assembled
D173A,D176A Mutant—The formation of a wild type preholo-
proteasome in which propeptides are retained could not be
detected in our real-time MS experiments, although this inter-
mediate must form prior to the active proteasome particle.

Therefore, a mutant of the Rhodococcus proteasome, altered in
its ability to assemble, was used to characterize this late-assem-
bly intermediate. Previously, we have reported that the β1-sub-
unit mutational variant, D173A,D176A, expressed recombi-
nantly in E. coli by using a bicistronic expression system, is
trapped at the preholoproteasome stage (25). The mass spec-
trum recorded for this mutant indicates that the major charge
state series corresponds in mass to preholoproteasomes,
whereas the minor charge state is assigned to half-proteasomes
(Fig. 6, A and B).

To examine in detail the composition of this late assembly
intermediate, preholoproteasomes formed using the D173A,
D176A mutant were subjected to in-source dissociation (Fig.
6C). Under these conditions of higher acceleration, series of
charge states are observed at higher m/z values than the pre-
holoproteasome. Two of these series are assigned to “stripped
complexes” that have lost one or two β1-subunits (Table 1). At
low m/z values, only α1-subunits are dissociated from the pro-
teasome, consistent with the architecture in which the two
α3-ring structures are exposed and in accord with previous
analyses of 20 S proteasomes (31, 33, 34). Expansion of the
peaks assigned to the stripped complexes, minus α1-subunits
or minus two α1-subunits, reveals fine splitting, indicating the
incorporation of various truncated forms of either the α1-sub-
units or the β1-subunits (Fig. 6, D and E). We could exclude the
possibility that the α1-subunits are cleaved as their mass meas-
ured from the low m/z region is consistent with the theoretical
mass. We therefore conclude that the β1-subunits are trun-
cated. By measuring the mass of the stripped complexes, we
could identify the statistical incorporation of the various

FIGURE 4. Real-time monitoring of the assembly pathway. ESI-MS spectra were taken after different time intervals at 37 (A) and 25 °C (B). At both temper-
atures, half-proteasomes with propeptides attached are formed initially. However, at 37 °C after 29 min, all the half-proteasome species have assembled into
intact processed proteasomes. An intermediate step in which both unprocessed half-proteasomes and intact proteasomes co-exist is observed after 25 min
(37 °C). By contrast, at 25 °C, half-proteasomes persist even after 5 days of assembly.

FIGURE 5. Mass spectrum of various dimers formed during the assembly
reaction. Shown is a spectrum recorded 12 min after starting the assembly
reaction at 37 °C. The same spectrum is shown in Fig. 3C but at a lower m/z
range. The charge states of dimers, labeled with stars, are indicated. Charge
states of the monomers are given alongside their labels (circles).
The spectra reveal that the intact preholoproteasome is composed of a minimum of 10 full-length \( \beta \)-subunits. Up to four of the \( \beta \)-subunits in any intact preholoproteasome, however, correspond to truncated \( \beta \)-subunits \( (\Delta 18) \). The fact that similar ratios of truncated subunits are observed within both stripped complexes implies that truncated subunits are not stripped preferentially. In summary, we have demonstrated that the \textit{in vivo} assembled preholoproteasome contains predominantly full-length \( \beta \)-subunits but that four of the 14 \( \beta \)-subunits correspond to truncated forms \( (\Delta 18) \).

**DISCUSSION**

In this study, we have followed the assembly of the \( \alpha \)- and \( \beta \)-subunits from \textit{R. erythropolis} into the 20 S proteasome,
using real-time MS. We were able to capture a transient intermediate along the in vitro reaction pathway, demonstrating that this early assembly intermediate is an α/β heterodimer. Such a heterodimer was predicted to exist (13); however, it has not been observed experimentally. Despite the high similarity in mass of the α/β-heterodimer and the α/α-homodimer, which prevents their distinction using biochemical methods, the high resolution of MS with the rapid time frame for recording spectra enabled the identification of this species. In the course of assembly, we could also follow the formation of unprocessed half-proteasomes and the gradual appearance of fully assembled holoproteasomes. This MS approach also allowed us to study in great detail the composition of the β-subunit propeptide during different stages of assembly. Interestingly, we could not detect preholoproteasomes that form from the docking of two unprocessed half-proteasomes during the in vitro assembly reaction. To investigate the composition of this late assembly intermediate, we used a mutational variant assembled in vitro. Overall, therefore, in this study, we were able to reveal the formation of both early and late intermediates and to demonstrate differential incorporation of truncated β-subunits within various stages of the assembly.

The observation that the first assembly intermediate for the Rhodococcus 20 S proteasome is an α/β-heterodimer is in contrast to that found for assembly of the Thermoplasma proteasome. The latter study showed that α-subunits spontaneously assemble into seven-membered rings when they are expressed alone in E. coli (16). Detailed comparison between the structures of proteasomes from Rhodococcus and Thermoplasma, however, suggests that there are fewer specific interactions and smaller contact areas between the α-subunits in the Rhodococcus proteasome (24). This may explain why extensive α-subunit interactions are observed in Thermoplasma but not in Rhodococcus. However, it remains to be established whether or not α-rings are assembly intermediates in vivo. This will depend upon the kinetics of the formation of the α/α homodimer versus that of the α/β heterodimer (35). If the formation of α/β heterodimers is much faster than assembly of α-rings, the assembly process of archaebacterial proteasomes would be expected to be similar to those of the eubacterial system studied here.

The preholoproteasome intermediate that forms from the dimerization of two half-proteasomes was not observed in our analyses indicates that it is short-lived. A similar conclusion was reported in a study that used electron microscopy to characterize late events in the Rhodococcus proteasome assembly (23). In an attempt to characterize this further intermediate, we analyzed a β1-subunit mutational variant. Using this mutant, we found that the preholoproteasome consists primarily of the full-length β1-subunit but that up to four of these β1-subunits are substituted by truncated β1-subunits (Δ18) (Fig. 6). This result demonstrates that the first 18 residues of the β1-subunit propeptide are not necessary for docking of two half-proteasomes.

Our results also demonstrate that the assembly process is temperature-dependent. Interestingly, however, assembly of the half-proteasome occurs at similar rates, even at the lower temperatures, whereas final docking of the two half-proteasomes is significantly retarded at the lower temperatures. To explain this observation, we assume that some structural perturbation within the individual β1-subunits is necessary for assembly to the full proteasome. More generally, it is established that protein-protein interfaces in obligate complexes, such as the proteasome, in which the monomers do not form stable structures in vivo, are generally more hydrophobic than non-oblige associations (36, 37). It may be speculated, therefore, that at the relevant physiological temperature, hydrophobic stretches are exposed. The accessibility of these hydrophobic regions may well be necessary for docking of the two half-proteasomes, explaining the favorable reaction at 37 °C and the hindered assembly at lower temperatures.

Detailed analysis of spectra of the β1-subunits has allowed us to gain insight into the mechanism of autocatalytic removal of the propeptide and its role in assembly. Previous observations suggested that the propeptide is degraded in a processive manner by undergoing multiple cleavages. In this study, we were able to identify clearly specific cleavage sites. In an attempt to find a common property of these 12 cleavage sites, we have examined in detail the location and relative abundance of the various degradation products (Fig. 7). A common feature of these residues is that they are all on the exposed face of the complex, which might explain the mechanism of their degradation. It is also noteworthy that 10 of the residues are polar, with the exception of Leu-43 and Gly-34, both of which are flanked by polar amino acids. This could also imply that in addition to exposed residues, processive degradation targets polar residues. Moreover, although the propeptide contains only one defined structural element, an α-helix positioned between Gly-34 and Asp-38, the cleavage sites are distributed both within and outside of this α-helix. We propose, therefore, that polarity and accessibility may well be important factors in determining favorable cleavage sites within the propeptide.

It is interesting to consider how the extent of cleavage affects the incorporation of truncated subunits into the intact proteasome and various assembly intermediates. The x-ray structure of a processing-incompetent Rhodococcus proteasome indicated the importance of the central box region (Ser-42 to Pro-27) to assembly (24) (Fig. 3E). Within this region, there are two highly conserved serine residues, Ser-42 and Ser-41, which form hydrogen bonds to Arg-85 of the α-subunit and Arg-82 of the β1-subunit, respectively. These interactions position a short proceeding α-helix (Gly-34 to Asp-38) and allow docking of the N-terminal end of the propeptide linking the β-subunit to two adjacent α-subunits. In accord with this structural information, our results indicate that once residues within the central box region are degraded, assembly is much slower or even abolished. We found heavily truncated subunits undergoing mul-
tible cleavage (18, 23, 38) trapped within α/β heterodimers or remaining monomeric at the end of the assembly reaction. Overall, it seems that longer propeptide sequences increase α/β-interfaces. This, in turn, promotes efficient assembly.

The observation that even the heavily truncated species (Δ50, Δ48, Δ42) were incorporated into the half-proteasome was surprising (Fig. 3D). The fact that these truncated variants were not incorporated into the full proteasome, however, implies that these mutations affect the correct docking of the half-proteasomes. In addition, it is noticeable that the degree of propeptide degradation is different between in vivo and in vitro assemblies. Although all the intermediates analyzed here for the in vitro assembly are degraded up to the first 18 residues (C-terminus to Ser-48) (Fig. 6 and Ref. 25), in vitro, the propeptide is more heavily truncated, and degradation can proceed up to the first 50 residues (C-terminus to Thr-16). This observation may imply that in vivo the α- and β-subunits are expressed, folded, and assembled in concert, thus preventing substantial degradation of the propeptide.

In summary, the study presented here reveals many of the different steps along the assembly pathway of the 20 S proteasome. The unique ability of MS to identify in great detail the modifications of individual β-subunits, differing by only one residue, has enabled us to monitor those that are trapped within the various intermediate states. We are therefore able to identify specific cleavage sites along the propeptide and to demonstrate that in vivo, the propeptide is significantly more protected from degradation than in vitro. More generally, this study highlights the tremendous potential for using real-time mass spectral acquisition and the phase transition from solution to gas phase to capture transient intermediates along assembly pathways of macromolecular complexes.

REFERENCES
1. Coux, O., Tanaka, K., and Goldberg, A. L. (1996) Annu. Rev. Biochem. 65, 801–847
2. Voges, D., Zwickl, P., and Baumeister, W. (1999) Annu. Rev. Biochem. 68, 1015–1068
3. Gerards, W. L., de Jong, W. W., Boelens, W., and Bloemendal, H. (1998) CMLS Cell. Mol. Life Sci. 54, 253–262
4. Dahlnann, B., Kopp, F., Kuehn, L., Niedel, B., Pfeifer, G., Hegerl, R., and Baumeister, W. (1989) FEBS Lett. 251, 125–131
5. Zwickl, P., Lottscheis, F., and Baumeister, W. (1992) FEBS Lett. 312, 157–160
6. Unno, M., Mizushima, T., Morimoto, Y., Tomisugi, Y., Tanaka, K., Yasuoka, N., and Tsukihara, T. (2002) Structure (Camb.) 10, 609–618
7. Groll, M., Ditzel, L., Lowe, J., Stock, W., Bochtler, M., Bartunik, H. D., and Huber, R. (1997) Nature 386, 463–471
8. Rock, L. I., and Goldberg, A. L. (1999) Annu. Rev. Immunol. 17, 739–779
9. Zuhl, F., Tamura, T., Dolenc, I., Cetkovic, Z., Nagy, I., De Mot, R., and Baumeister, W. (1997) FEBS Lett. 400, 83–90
10. Kruiger, E., Kloetzle, P. M., and Enenkel, C. (2001) Biochimie (Paris) 83, 289–293
11. Seemuller, E., Zwickl, P., and Baumeister, W. (2001) in The Enzymes, Vol. XXII, 3rd Ed., pp. 335–371, Academic Press, Orlando, FL
12. Seemuller, E., Lupas, A., and Baumeister, W. (1996) Nature 382, 468–471
13. Zuhl, F., Seemuller, E., Golbik, R., and Baumeister, W. (1997) FEBS Lett. 418, 189–194
14. Wilson, H. L., Ou, M. S., Aldrich, H. C., and Maupin-Furlow, J. (2000) J. Bacteriol. 182, 1680–1692
15. Maupin-Furlow, J. A., Aldrich, H. C., and Ferry, J. G. (1998) J. Bacteriol.
16. Zwickl, P., Kleinz, J., and Baumeister, W. (1994) Nat. Struct. Biol. 1, 765–770
17. Nagy, I., Tamura, T., Vanderleyden, J., Baumeister, W., and De Mot, R. (1998) J. Bacteriol. 170, 5448–5453
18. Schmidtke, G., Kraft, R., Kostka, S., Henklein, P., Frommel, C., Lowe, I., Huber, R., Kloetzel, P. M., and Schmidt, M. (1996) EMBO J. 15, 6887–6898
19. Heinemeyer, W., Fischer, M., Krimmer, T., Stachon, U., and Wolf, D. H. (1997) J. Biol. Chem. 272, 25200–25209
20. Chen, P., and Hochstrasser, M. (1996) Cell 86, 961–972
21. Groll, M., Brandstetter, H., Bartunik, H., Bourenkow, G., and Huber, R. (2003) J. Mol. Biol. 327, 75–83
22. Grziwa, A., Maack, S., Puhler, G., Wiegand, G., Baumeister, W., and Jaenicke, R. (1994) Eur. J. Biochem. 233, 1061–1067
23. Mayr, J., Seemuller, E., Muller, S. A., Engel, A., and Baumeister, W. (1998) J. Struct. Biol. 124, 179–188
24. Kwon, Y. D., Nagy, I., Adams, P. D., Baumeister, W., and Jap, B. K. (2004) J. Mol. Biol. 335, 233–245
25. Witt, S., Kwon, Y. D., Sharon, M., Felderer, K., Beuttler, M., Robinson, C. V., Baumeister, W., and Jap, B. K. (2006) Structure (Camb.) 14, 1179–1188
26. van den Heuvel, R. H., and Heck, A. J. (2004) Curr. Opin. Chem. Biol. 8, 519–526
27. Benesch, J. L., and Robinson, C. V. (2006) Curr. Opin. Struct. Biol. 16, 245–251
28. Fandrich, M., Tito, M. A., Leroux, M. R., Rostom, A. A., Hartl, F. U., Dobson, C. M., and Robinson, C. V. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 14151–14155
29. Remaut, H., Rose, R. J., Hannan, T. J., Hultgren, S. J., Radford, S. E., Ashcroft, A. E., and Waksman, G. (2006) Mol. Cell 22, 831–842
30. Sobott, F., Hernandez, H., McCammon, M. G., Tito, M. A., and Robinson, C. V. (2002) Anal. Chem. 74, 1402–1407
31. Chernushevich, I. V., and Thomson, B. A. (2004) Anal. Chem. 76, 1754–1760
32. Hernandez, H., and Robinson, C. V. (2007) Nat. Protocols 2, 715–726
33. Sharon, M., Witt, S., Felderer, K., Rockel, B., Baumeister, W., and Robinson, C. V. (2006) J. Biol. Chem. 281, 9569–9575
34. Loo, J. A., Berhane, B., Kaddis, C. S., Wooding, K. M., Xie, Y., Kaufman, S. L., and Chernushevich, I. V. (2005) J. Am. Soc. Mass Spectrom. 16, 998–1008
35. Heinemeyer, W., Ramos, P. C., and Dohmen, R. J. (2004) CMLS Cell. Mol. Life Sci. 61, 1562–1578
36. Lo Conte, L., Chothia, C., and Janin, J. (1999) J. Mol. Biol. 285, 2177–2198
37. Jones, S., and Thornton, J. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13–20
38. Akopian, T. N., Kisselev, A. F., and Goldberg, A. L. (1997) J. Biol. Chem. 272, 1791–1798
39. Sobott, F., McCammon, M. G., and Robinson, C. V. (2003) Int. J. Mass Spectrom. 230, 193–200