Proteasome function is dispensable under normal but not under heat shock conditions in *Thermoplasma acidophilum*

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Abstract Hitherto the biology of proteolysis in prokaryotes, particularly in archaea, is only poorly understood. We have used the tri-peptide vinyl sulfone inhibitor carboxybenzyl-leucyl-leucyl-leucine vinyl sulfone (Z-L_3VS) to study the in vivo function of proteasomes in *Thermoplasma acidophilum*. Z-L_3VS is a potent inhibitor of the *Thermoplasma* proteasome and is capable of modifying 75 to 80% of the proteasomal β-subunits in cell cultures. Inhibition of proteasomes has only marginal effects under normal growth conditions. Under heat shock conditions, however, the effects of proteasome inhibition are much more severe, to the extent of complete cell growth arrest. These data suggest that other proteolytic systems may exist that can compensate for the loss of proteasome function in *T. acidophilum*.

Key words: Proteasome; Inhibitor; *Thermoplasma acidophilum*; Archaea

1. Introduction

Intracellular protein degradation is essential for the maintenance of homeostasis, it serves to remove abnormal or misfolded proteins and is a key element of many cellular regulatory mechanisms. A large, ubiquitous (2 mDa) ATP-dependent proteolytic complex, the 26S proteasome performs these tasks [1].

The 26S complex consists of two asymmetric 19S 'caps' flanking a barrel-shaped core 20S proteasome complex of about 700 kDa. Collectively, four seven-membered rings of subunits make up this core complex; the α-type subunits form the two outer rings and the β-type subunits the two inner rings. The 20S complex is traversed from end to end by a channel which widens into three large inner cavities. The central cavity, which is bounded by the two β-subunit rings, harbors the active sites; the N-terminal threonine of the β-subunits acts as the catalytic nucleophile (for reviews see [2,3]). Substrates enter the proteasome via a narrow channel at the center of the α-subunit rings. Thus the 20S proteasome not only executes the cleavage of substrates, it also confines proteolytic activity to an inner nanocompartment, a principle which is of particular importance for prokaryotic cells lacking inner membrane-bounded organelles [4].

The structure and function of the 19S caps which associate with the two termini of the 20S core is only poorly defined. The 19S caps are built from approximately 15 different subunits with molecular masses of 25–110 kDa; six of them are ATPases belonging to the AAA (ATPases Associated with a variety of cellular Activities) family (for recent reviews see [5,6]). It is plausible, but not proven that these ATPases form a ring-shaped interface to the 20S core complex and serve to unfold substrate before it is translocated into the inner cavities of the proteolytic core enzyme. Other subunits of the cap complex are implicated in the recognition of substrate proteins carrying degradation signals and thus confer selectivity upon degradation by the proteasome. In eukaryotes the attachment of ubiquitin to a target protein is seen as the principal mechanism by which proteins are marked for degradation [7,8].

Before 20S proteasomes were discovered in *Thermoplasma acidophilum* [9] it was assumed that their occurrence was restricted to eukaryotes. In the meantime, genes of 20S proteasomes were found in all archaebacterial genomes sequenced so far (*Methanococcus jannaschii*, *Archaeoglobus fulgidus*, *Methanobacterium thermoautotrophicum*), and also in a group of eubacteria, the actinomycetes [10]. Recently, in the archaebacterium *M. jannaschii* a gene was found which is homologous to the ATPase subunits of the 19S cap complex [11]. It remains to be established that this ATPase forms a complex with the 20S proteasome and renders proteolysis ATP-dependent. Also in the actinomycetes *Rhodococcus erythropolis* and in *Mycobacterium leprae* ATPase genes of the AAA family were found in close proximity to the proteasome genes [12]. This may indicate that also in eubacteria the proteasomal pathway of protein degradation is energy-dependent. At present, the biology of proteolysis in prokaryotes is only poorly understood.

Key to elucidating proteasome function is the ability to manipulate their activity in vivo. Currently several classes of small molecule inhibitors of the proteasome have been described which can block protein degradation. Peptide aldehydes act by forming a transient covalent bond with the active site threonine of proteasomal β-subunits. Carbobenzoxyl-leucinyl-leucinyl-leucinal (MG132) and carbobenzoxyl-leucinyl-leucinyl-norvalinal (MG115) have shown to be potent proteasome inhibitors both in vitro and in living cells [13,14]. The breakdown of short-lived proteins in *Saccharomyces cerevisiae* was inhibited as much as 70–80% in the presence of MG132. These agents, however, are not specific for proteasomes and therefore caution must be taken when interpreting results obtained with these inhibitors. A more specific proteasome inhibitor is the *Streptomyces* metabolite lactacystin [15,16] which modifies the active site threonine of eukaryotic β-subunits by irreversible alkylation of the nucleophilic hydroxyl. However, this reagent is not a potent inhibitor of archaeal proteasomes.

Recently a new class of proteasome inhibitors, the peptide

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Abbreviations: OD, optical density; DMSO, dimethyl sulfoxide; NMec, 7-amino-4-methycoumarin; Suc, succinyl

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vinyl sulfones, has been described [17]. Carboxybenzyl-leucyl-leucyl-leucine vinyl sulfone (Z-L_3VS) inhibits the chymotrypsin-like, trypsin-like, and peptidylglutamyl peptidase activities of the proteasome both in vivo and in vitro. As with lactacystin the modification of the active site threonine is irreversible and covalent. Using a ^{125}I-labeled derivative it was demonstrated that only proteasomal β-subunits were modified in crude extract from *T. acidophilum* [17].

In this communication we describe experiments using the inhibitor Z-L_3VS for in vivo studies with *T. acidophilum*. We study the effects of proteasome inhibition under normal growth conditions as well as under conditions of heat shock induced stress.

2. Materials and methods

2.1. Culture conditions

T. acidophilum was grown in the medium originally described by Freundt [18] with 0.2% yeast extract (Difco) as a carbon source. The pH was adjusted to pH 1.5 with H_2SO_4. Usually 25 ml cultures were incubated in Erlenmeyer flasks kept in an oil bath shaker with 120 rpm at 58°C. In order to perform heat shock experiments cultures were shifted from 58°C to 74°C for 2 h, if not otherwise mentioned. Z-L_3VS was added to the medium 1 h before exposing the cells to heat shock conditions. The cell density was monitored with a spectrophotometer by measuring aliquots of the *Thermoplasma* cultures at OD_{540}.

2.2. Purification of proteasomes

To measure the modification of the proteasomes by Z-L_3VS *T. acidophilum* was grown to OD_{540}=0.9 at 58°C and pH 1.5. Subsequently the cell culture was incubated for 3 h in the presence of 1% DMSO and 15 μM Z-L_3VS. Cells were harvested and resuspended in 3 volumes of 0.1 M HEPES buffer, pH 8.0. Crude extract was obtained by sonification of the cells and centrifugation at 20 000×*g* for 30 min. The crude extract was dialyzed two times against 10 mM potassium-phosphate buffer, pH 7.0. The suspension was adjusted at 100 mM potassium-phosphate, pH 7.0 and applied to a hydroxyapatite column with a size of 7×0.7 cm. The flow rate was 20 ml per hour during the whole procedure. The column was washed with 100 mM potassium-phosphate, pH 8.0 until the base line was reached. The proteasomes were eluted using a 200–600 mM phosphate linear gradient. The fractions were assayed for proteolytic activity with the fluorigenic peptide substrate Suc-Leu-Leu-Val-Tyr-NMec. Proteins of the most active fractions were examined by SDS-polyacrylamide gel electrophoresis. Fractions containing highly purified proteasomes were pooled and used for LC/MS and enzyme activity analysis.

2.3. LC mass spectrometry

The protein solution was applied to a reversed phase HPLC coupled on-line to an atmospheric pressure ionization source fitted to the tandem quadrupole instrument API 365 (Sciex, Thornhill, Ontario, Canada). The instrument m/z scale was calibrated with the ammonium adduct ions of polypropylene glycol (PPG). The average molecular mass values of the proteins were calculated from the m/z peaks in the charge distribution profiles of the multiple charged ions [19]. The deconvoluted mass spectra provide the final estimated masses.

3. Results

3.1. Modification of Thermoplasma proteasomes by Z-L_3VS

Thermoplasma thrives at 60°C and an acidic environment as low as pH 1. To make sure that the proteasome inhibitor Z-L_3VS is stable under these growth conditions, it was incubated for 2 h in growth medium (60°C; pH 1.5) followed by neutralization. Neither the temperature nor the low pH affected the inhibitory potency of Z-L_3VS (data not shown).

A culture of *Thermoplasma* was incubated with 15 μM inhibitor for 4 h and the amount of modified β-subunits determined by LC/MS. Modification of the NH_2-terminal threonine by Z-L_3VS results in a shift of the mass from 22271 Da (unmodified) to 22822 Da (modified). As shown in Fig. 1 about 80% of the active site threonine residues are modified by Z-L_3VS. Measurement of chymotryptic activity gave a rate of 278 nmol h^{-1} mg^{-1} for the proteasomes from cells which were incubated with Z-L_3VS; the specific activity for unmodified proteasomes is 1100 nmol h^{-1} mg^{-1}. A reduction of peptidase activity by about 75% is in good agreement with the data obtained by LC/MS. Longer periods of growth in the presence of the inhibitor did not lead to a more complete inhibition of proteasomes.

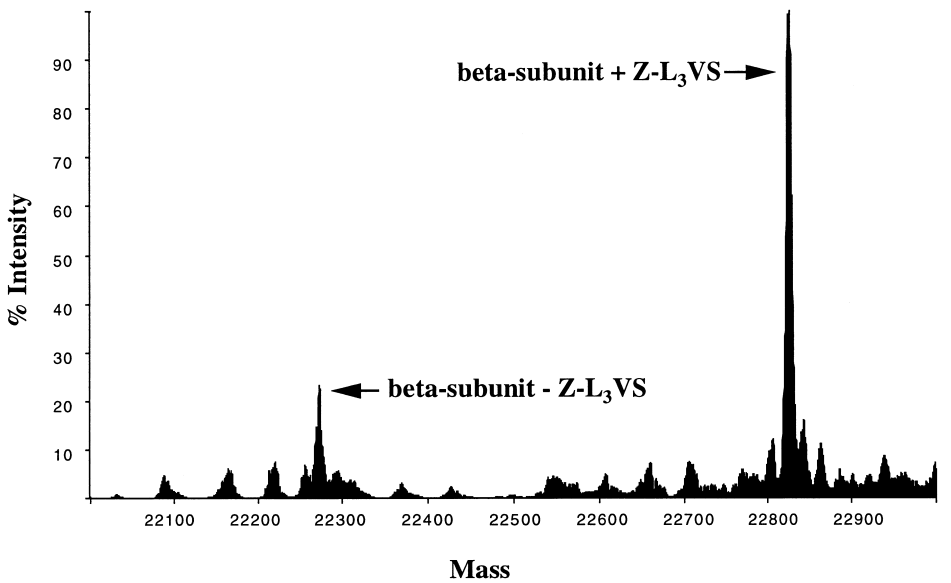

Fig. 1. LC/MS run of purified proteasomes. Proteasomes were isolated from a *T. acidophilum* culture which was incubated with 15 μM of the proteasome inhibitor Z-L_3VS. The signals at 22271 Da and 22822 Da correspond to unmodified and modified proteasome β-subunits, respectively. On the graph the molecular mass (Mass) is plotted against the relative intensity of the signal.

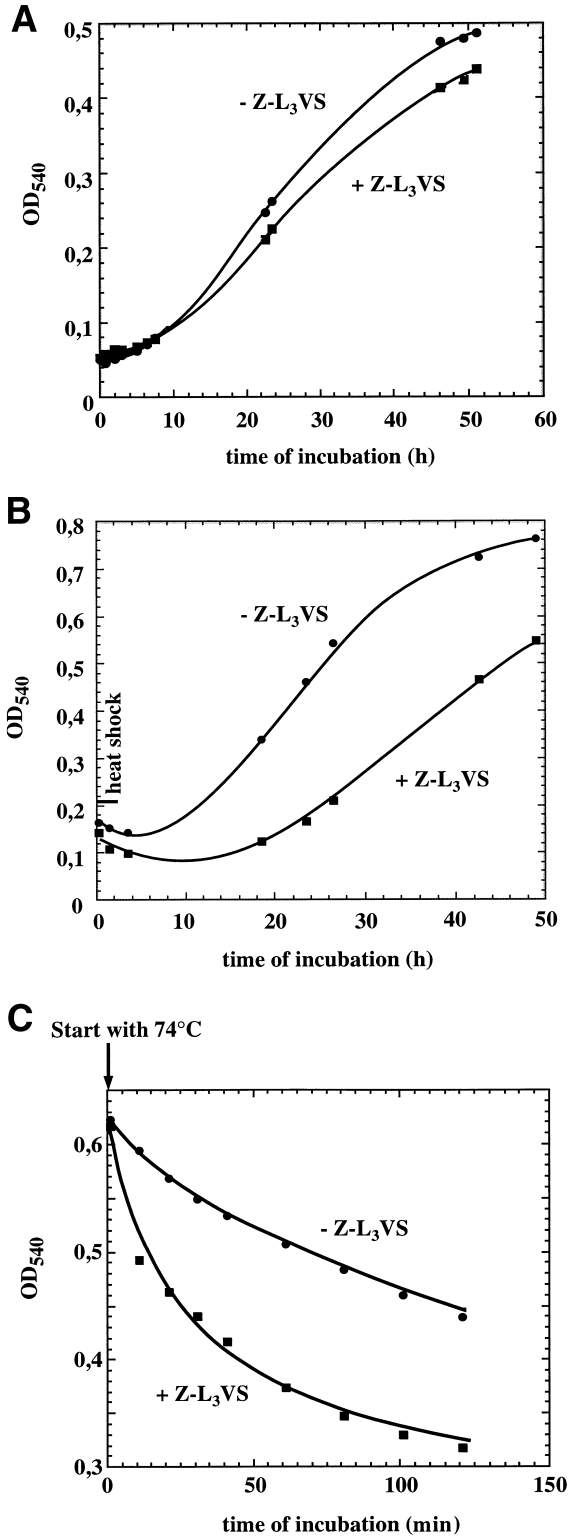

Fig. 2. Growth of *T. acidophilum* cultures in the presence (10 μM) and absence of the proteasome inhibitor Z-L_3VS, respectively. Cells were grown at 58°C, pH 1.5 as described in [18] unless otherwise mentioned. To increase the solubility of the medium for the hydrophobic proteasome inhibitor, DMSO was added to a final concentration of 1%. *T. acidophilum* cultures were grown at 58°C (A). Cell cultures were exposed to heat shock conditions for 2 h. Afterwards the growth temperature was again adjusted to 58°C (B). *T. acidophilum* cultures grown to an OD_{540} of 0.65 were constantly kept at 74°C (C). Incubation time is plottet against cell density (OD_{540}).

3.2. Growth of T. acidophilum in the presence of Z-L_3VS

Cultures of *Thermoplasma* grown under optimal conditions were incubated with increasing concentrations of the proteasome inhibitor Z-L_3VS. As shown in Fig. 2A the presence of 10 μM Z-L_3VS resulted in a slight but significant increase (about 10%) of the doubling time of the cells from 16 h to 18 h. Higher concentrations of proteasome inhibitor did not further reduce cell growth.

The heat shock experiments with *Thermoplasma* were performed by shifting cultures to 74°C for 2 h. Cell density decreased early during heat shock both in the presence and absence of L_3VS. However, in contrast to the control cells L_3VS treated cultures arrested cell growth for up to one day after administration of heat shock (Fig. 2B). In other experiments where the heat shock periods were reduced, the lag phase of the cell growth was proportionally reduced (data not shown).

To get a more precise picture of the decrease in cell density at the beginning of the heat shock period, cultures were grown to an OD_{540} of 0.65 in the presence or absence of L_3VS and shifted to 74°C for several hours (Fig. 2C). Without Z-L_3VS, cell density of the culture decreased constantly with a half time of about 4 h. Upon L_3VS treatment, cell density in the culture decreased more rapidly, with the sharpest decline in the first 10 min of heat shock. After 3 h at elevated temperature, aliquots from both cultures were transferred into fresh medium and the temperature shifted back to 58°C. After 1 day the cells from the control culture began to grow while the Z-L_3VS treated cells were no longer viable.

4. Discussion

In the present study we have shown that under normal growth conditions inhibition of proteasomes in *Thermoplasma* by Z-L_3VS does not result in a loss of viability; the observed decrease of growth rates is rather marginal. With the caveat that inhibition is not complete we conclude that under these conditions proteasome activity is not essential. This is in accordance with recent deletion studies performed with the eubacterium *Mycobacterium smegmatis*: mutants lacking the 20S proteasome did not show impaired growth, neither under normal nor under stress conditions [20]. It is, however, in stark contrast to the situation in yeast where null mutations of all except one of the genes of proteasomal subunits are lethal [21], suggesting that prokaryotes but not eukaryotes possess redundant proteolytic systems which can complement each other (see below).

Interestingly, inhibition of the proteasome during periods of stress had a dramatic effect on the growth of *Thermoplasma* cells and the extended exposure of Z-L_3VS treated cells to high temperature resulted in a permanent loss of viability. Under heat shock conditions cells are exposed to an increased level of unfolded proteins. These polypeptides expose hydrophobic amino acid side chains which tend to form intramolecular and intermolecular aggregations [22]. To avoid these unproductive interactions, unfolded proteins either must be kept soluble by chaperones or must be degraded.

Prokaryotic cells, archaea and bacteria, possess multiple proteolytic systems, some of them energy-dependent and some energy-independent, which could be employed in quality control [23]. It is at present unknown what the cellular substrates of the various proteolytic systems are and to what

extent the spectra of substrates overlap. *Thermoplasma* has at least two major proteolytic systems besides the proteasome: protease La/Lon (ATP-dependent) (unpublished results) and Tricorn protease (ATP-independent) [24]. These proteases might act as 'back-up' systems capable of complementing the loss of proteasome activity. This could allow *Thermoplasma* to survive without functional proteasomes under non-stress conditions. Only under stress conditions, that is when the level of misfolded proteins will become very high, blockage of the proteasome-dependent degradation pathway results in growth arrest. Our finding with *Thermoplasma* is in line with the recent observation that mammalian cells can grow in presence of high concentrations of a vinyl sulfone proteasome inhibitor [25]. These cells overcome the loss of proteasome function by employing a secondary, ATP-independent proteolytic system which may compensate by performing many of the critical metabolic functions of the proteasome.

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