

## Characterization of the proteasome from the extremely halophilic archaeon *Haloarcula marismortui*

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**Summary** A 20S proteasome, comprising two subunits  $\alpha$  and  $\beta$ , was purified from the extreme halophilic archaeon *Haloarcula marismortui*, which grows only in saturated salt conditions. The three-dimensional reconstruction of the *H. marismortui* proteasome (Hm proteasome), obtained from negatively stained electron micrographs, is virtually identical to the structure of a thermophilic proteasome filtered to the same resolution. The stability of the Hm proteasome was found to be less salt-dependent than that of other halophilic enzymes previously described. The proteolytic activity of the Hm proteasome was investigated using the malate dehydrogenase from *H. marismortui* (HmMalDH) as a model substrate. The HmMalDH denatures when the salt concentration is decreased below 2 M. Under these conditions, the proteasome efficiently cleaves HmMalDH during its denaturation process, but the fully denatured HmMalDH is poorly degraded. These in vitro experiments show that, at low salt concentrations, the 20S proteasome from halophilic archaea eliminates a misfolded protein.

**Keywords:** archaeobacteria, halophiles, stress.

### Introduction

Extreme halophilic microorganisms thrive in ecological niches such as the Great Salt Lakes or the Dead Sea, where the salt concentration is close to saturation. Halophilic archaeobacteria accumulate KCl to multi-molar concentrations (over 3 M) in their cytosol to compensate for the external osmotic pressure (Ginzburg et al. 1970). Most halophilic proteins denature when the salt concentration decreases, whereas high concentrations of salt normally alter non-halophilic protein stability and solubility (Timasheff 1993, Madern et al. 2000). Therefore, to remain stable and soluble in saturated saline conditions, halophilic proteins must have developed adaptative mechanisms. The main characteristic of soluble halophilic enzymes is their high content of acidic residues (Eisenberg et al. 1992). High-resolution structures of three halophilic proteins show that the acidic residues are localized in patches on the protein surface (Dym et al. 1995, Frolow et al. 1996, Pieper et

al. 1998). An extensive study of malate dehydrogenase from *Haloarcula marismortui* has led to the conclusion that hydrated salt ions are associated with acidic residues situated on the protein surface in such a way that the salt concentration of the solvation layer is higher than that of the bulk solvent (Bonnete et al. 1994, Madern et al. 1995, Ebel et al. 1999). It was suggested that this characteristic allows halophilic proteins to remain stable and soluble in high salt concentrations (Zaccai et al. 1989). This structural adaptation to extreme salt conditions implies that variation in salt concentration in the environment represents a severe stress for extreme halophilic organisms. Hence, in the crowded cytosolic environment, misfolded proteins generated by low-salt stress may aggregate and cause cell death (Ellis and Hartl 1999).

It has been proposed that, in archaea, the 20S proteasome, which constitutes the core of the central protease in the protein degradation pathway (Dahlmann et al. 1989, Baumeister et al. 1998), could be responsible for the nonspecific elimination of abnormal proteins generated by various stresses, in addition to its role in controlling the rate of protein turnover (Maupin-Furlow et al. 2000, Sherman and Goldberg 2001). The structure of the 20S proteasome from the archaeon *Thermoplasma acidophilum* has been extensively studied (Zwickl et al. 1992, Lowe et al. 1995, Lupas et al. 1997) and found to represent a simplified version of those in eukaryotic systems. In archaea, the 20S proteasome consists of only two types of subunits ( $\alpha$  and  $\beta$ ) arranged in four stacked rings of seven subunits each, whereas the eukaryotic proteasome contains seven distinct subunits (Coux et al. 1996, Baumeister et al. 1998). The two outer rings of the 20S proteasome are composed of  $\alpha$ -type subunits, whereas the two inner rings are composed of  $\beta$ -type subunits, which bear the proteolytic active sites (Seemuller et al. 1995). The proteasome eliminates the majority of proteins in a processive mechanism generating small peptides (Akopian et al. 1997, Kisselev et al. 1999). However, the initial process that leads to the degradation of proteins in a cell is still unclear. The process is energy dependent and consists of recognizing the protein to be degraded and then rendering the substrate accessible to the compartmentalized active sites of the proteasome (Schmidt et al. 1999). The energy-dependent recognition step

is believed to control the destruction of the proper proteins. In eukaryotes, the proteins to be degraded are first tagged by ubiquitin (Hershko and Ciechanover 1998). Such ubiquitin targeting systems have not been identified in prokaryotes, and little is known about how native substrate proteins are recognized for degradation in archaea. In prokaryotes and eukaryotes, the native protein targeted for degradation by self-compartmentalizing proteases is recognized by other regulatory complexes that can also act as molecular chaperones (Horwich et al. 1999). In eukaryotes, the 19S cap is a multi-subunit complex that associates with the 20S proteasome to form the energy-dependent 26S proteasome (Coux et al. 1996). The base domain of the 19S cap comprises six subunits that form a ring-shaped complex that possesses ATPase activity and belongs to the AAA (ATP:ADP antiporter) family (Russell et al. 1996, Patel and Latterich 1998). In archaea, a homolog of this 26S eukaryotic proteasome activating complex, called PAN (proteasome activating nucleotidase), has been characterized (Zwickl et al. 1999, Wilson et al. 2000). These complexes are believed to recognize exposed hydrophobic surfaces of improperly folded proteins and display unfoldase (reverse chaperone) activity that triggers protein degradation (Glickman et al. 1998, Wickner et al. 1999, Benaroudj and Goldberg 2000).

Because of their specific adaptation to extreme conditions (e.g., temperature, salt, pressure and pH), archaeal cells represent appropriate model systems to study the environmental stress response. Little is known about the biological significance of the 20S archaeal proteasome in the stress response and substrate proteins have not been identified. It is likely that ATP-dependent regulatory complexes, such as PAN, are indispensable for the elimination of abnormal proteins (Sherman and Goldberg 2001). However, in microorganisms, the 20S proteasome can function autonomously in the efficient degradation of misfolded proteins in the cell during extreme environmental stress. The objective of this work was to study *in vitro* the stability and proteolytic activity of the proteasome in *H. marismortui*.

A proteasome from *Haloferax volcanii* was previously characterized by Wilson et al. (1999). Compared with *H. marismortui*, *H. volcanii* is a moderate halophile that requires a lag period and synthesis of specific, high-salt-related proteins to adapt to hypersaline conditions (Mullakhanbhai and Larsen 1975, Mojica et al. 1997). The function of the *H. volcanii* proteasome was found to be salt dependent. However, the method used to assess the *in vitro* activity did not discriminate between the salt concentration needed for enzymatic activity and that needed for stability. Here, we studied independently the catalytic activity and the stability of the proteasome from *H. marismortui* (Hm proteasome). Malate dehydrogenase from *H. marismortui* (HmMalDH) was found to be a good substrate in protease assays and we studied the proteasome activity in low salt concentration. We also obtained the three-dimensional (3-D) structure for the Hm proteasome by image reconstruction from negatively stained electron micrographs and compared it with the X-ray crystal structure of its homolog

from *T. acidophilum*. Our results provide new insight into the functioning of the 20S archaeal proteasome.

## Materials and methods

### *Strains, media and culture conditions*

*Haloarcula marismortui* cells were kindly provided by A. Oren (University of Jerusalem). Cells were cultivated in 2-l flasks at 37 °C with gentle agitation in a growth medium containing 3.5 M NaCl (Mevarech et al. 1977). Cells were harvested at  $OD_{660} = 2$  by centrifugation for 1 h at 12,000 g and stored at -80 °C until use.

### *Purification of the H. marismortui proteasome*

Cell pellets (usually corresponding to 30 l of culture) were thawed and suspended in one volume of Buffer A (50 mM Tris-HCl, 2.2 M  $(NH_4)_2SO_4$ , 40 mM  $MgAc_2$ , pH 7.6) containing 20 mg of grade II DNaseI (Boehringer Mannheim). After 30 min of agitation at room temperature, aliquots of the cell lysate were homogenized by ultrasonication ( $6 \times 10$  s). The cell extract was centrifuged for 60 min at 30,000 g. The supernatant was centrifuged for 2 h at 160,000 g. After a 48-h dialysis against Buffer A at 4 °C, the S160 supernatant was then mixed batchwise with 400 ml of Sepharose 4B (Pharmacia) equilibrated in Buffer A. Note that Sepharose 4B is used as a hydrophobic interaction column in halophilic protein purification processes. After a 2-h incubation at room temperature, the gel was packed in a  $5.5 \times 30$  cm column and protein was eluted based on hydrophobicity with a linear ammonium sulfate ( $(NH_4)_2SO_4$ ) gradient (2.2–0.4 M in 950 ml of Buffer A). *Haloarcula marismortui* cells were found to contain a strong endogenous peptidase activity in the first stages of the purification that interfered with the detection of the proteasome chymotrypsin-like activity when using a typical fluorogenic substrate. Moreover, the protein fractions contained a substance that fluoresced when excited at 360 nm. We therefore used electron microscopy to look for rod-shaped particles that resembled proteasomes in the protein extracts. Fractions containing both rod-shaped particles and chymotrypsin-like activity, obtained at approximately 0.6 M  $(NH_4)_2SO_4$ , were pooled and solid ammonium sulfate was added to a concentration of 1.9 M. The sample was applied to a DE52 cellulose column ( $2.6 \times 25$  cm) equilibrated in 50 mM Tris, 2 M  $(NH_4)_2SO_4$ , pH 7.6. Proteins were eluted with a decreasing  $(NH_4)_2SO_4$  gradient (1.9–0.5 M) and an increasing NaCl gradient (0–2 M) over 500 ml. The active fractions, obtained at 0.8 M NaCl and 1.2 M  $(NH_4)_2SO_4$ , were pooled and dialyzed against 20 mM sodium phosphate buffer containing 4 M NaCl. The protein sample was applied to a column ( $1.5 \times 10$  cm) of hydroxyapatite (BioRad) pre-equilibrated with dialysis buffer. The column was developed at  $18 \text{ ml h}^{-1}$  with a 200-ml linear phosphate gradient from 20 to 300 mM phosphate in 4 M NaCl. The fractions containing the proteasome subunits were dialyzed against 2 M KCl in 20 mM Tris-HCl, pH 7.6. The sample was applied to a G4000 (TosoHaas, Montgomeryville, PA) gel filtration column mounted on an HPLC system. The elution

buffer was 2 M KCl in 20 mM Tris-HCl, pH 7.6 at 0.9 ml min<sup>-1</sup>. Protein concentration was determined by the method of Bradford with bovine serum albumin (BSA) as the standard (Bradford 1976). The SDS-polyacrylamide gel electrophoresis (12%) was performed according to Laemmli (1970).

#### *Peptidase assays*

Chymotrypsin-like activity was assayed fluorimetrically following the release of 7-amino-4-methylcoumarin (AMC) from the synthetic peptide succinyl-Leu-Leu-Val-Tyr-AMC (Bachem, Bubendorf, Switzerland). The assay mixture was prepared by dissolving the fluorogenic peptide (10 mM in DMSO) at 100 nM final concentration in 20 mM Tris-HCl, pH 7.6, containing various KCl concentrations. Assays were initiated by addition of 2 µg of enzyme to 1 ml of preheated assay mixture. The hydrolysis of substrate was monitored in a continuous rate assay at different temperatures and in different salt conditions in a thermostatically controlled fluorimeter. Linear rates of increasing fluorescence intensity were recorded for up to 10 min at an excitation wavelength of 370 nm and emission of 460 nm. The fluorimeter was calibrated with AMC solutions prepared in the assay buffer. Specific activity is expressed as nmol of AMC released per min per mg of protein.

#### *Stability*

For stability measurements, 2.5 µg of purified proteasome was incubated for 24 h at 40 °C in 500 µl of assay buffer containing 20 mM to 2 M KCl. The residual activity was measured at 40 °C after the addition of one volume of preheated substrate peptide solution (final concentration of 100 mM) containing the appropriate amount of KCl to attain a final concentration of 0.5 M. Halophilic malate dehydrogenase stability was estimated in parallel experiments and the residual activity was measured as described by Cendrin et al. (1993).

#### *Degradation of halophilic malate dehydrogenase*

Recombinant halophilic HmMalDH was over-expressed in *Escherichia coli* and purified in an active state as described by Cendrin et al. (1993). The pure protein was stored at 4 °C in 3 M KCl, 50 mM Tris-HCl, pH 8. For the protease assay, the enzyme was diluted 20-fold in 20 mM Tris-HCl, pH 7.6 containing either 3 or 0.4 M KCl, to give a final enzyme concentration of 0.05 mg ml<sup>-1</sup>. Complete enzyme inactivation was reached in the 0.4 M KCl denaturing buffer after a 30-min incubation at 40 °C as monitored by the enzymatic assay described by Zaccari et al. (1989). Native or denatured malate dehydrogenase (0.1 µg) was mixed with 2.8 µg of proteasome in 100 µl of 20 mM Tris-HCl buffer, pH 7.6 containing different KCl concentrations (from 0.4 to 2 M). We used low-adhesion micro-tubes that were previously pre-quenched with BSA to avoid nonspecific retention of denatured malate dehydrogenase. The mixtures were incubated for 1 h at 40 °C. Reactions were stopped by addition of 200 µl of 20% trichloroacetic acid with 10 µg of tRNA as a carrier. Samples were centrifuged (30 min at 12,000 g) and pellets were washed

with cold acetone, dried and resuspended in 20 µl of 20 mM Tris-base. Proteins were resolved by SDS-PAGE using 15% polyacrylamide and blotted on a Hybond-P nitrocellulose membrane (Amersham) according to the manufacturer's protocol. The HmMalDH was immunodetected with rabbit polyclonal antibodies raised against the protein using the ECL detection kit from Amersham-Pharmacia.

#### *The 90° scattering experiments*

To follow HmMalDH aggregation, we used a spectrofluorimeter (SLM/Aminco, Champaign, IL) with an excitation and emission light of 400 nm. In the cuvette, we diluted either the denatured (in 0.4 M KCl) or the native (in 3 M KCl) HmMalDH to give a final protein concentration of 20 µg ml<sup>-1</sup> and for 2 h monitored light at 400 nm that was scattered at 90°.

#### *Electron microscopy*

We used negative staining rather than cryo-electron microscopy because of the high concentration of salt in the samples. Sample (0.1 mg ml<sup>-1</sup>) was absorbed to the clean side of a carbon film on mica, stained with 2% (w/v) ammonium molybdate, and transferred to a 400-mesh copper grid. Specimens were observed with a JEOL 1200 EX II microscope at 100 kV. Images were obtained under low dose conditions (< 100 e<sup>-</sup> per nm<sup>2</sup>) at a nominal magnification of 40,000× with a defocus chosen so that the first zero of the contrast transfer function (CTF) was beyond 2.0 nm<sup>-1</sup>. The images were recorded on KODAK SO163 film and developed in 1/3 strength KODAK D19 developer for 9 min at room temperature.

#### *Image processing and 3-D reconstruction*

Image quality was checked on an optical bench and 10 selected images were digitized on an Optronics microdensitometer, with a step size of 25 µm corresponding to a pixel size of 0.625 nm on the specimen. Particle selection was performed with the MRC program, Ximdisp (Crowther et al. 1996). A total of 1000 side views and 150 top views of the particles were selected, band pass filtered between 25–2 nm without CTF correction, and normalized to the same mean and standard deviation with SPIDER (a system for processing image data in electron microscopy and related fields; Wadsworth Center, NY). Multivariate statistical analysis in SPIDER was used in the initial analysis to determine the symmetry of the top views. This preliminary analysis gave unambiguously 7-fold symmetry for the macromolecule (data not shown). We used only the side views because they give full 3-D information when they are combined with 7-fold symmetry. The image analysis was performed with only the asymmetric unit of the macromolecule (1/7 of 360°) (Roseman et al. 1996). We generated a preliminary 3-D model by back projection, applying 7-fold symmetry to a single centered side view. This model was re-projected in side views and low tilted (10 and 20°) side views with the same azimuthal distribution (5°). All the images were aligned against the projections of this model. The images that matched with one angular class were summed and the mean and variance were calculated. The average images of the side



views with a good variance map were used to generate a new model by iterative back projection. This reconstruction was used as a new model and five iterative cycles were performed with the same azimuthal distribution. Because the model converged, we increased the azimuthal distribution to 52 (one projection every degree). After 20 more cycles the model and class distribution were stable and the refinement was complete. Ten to 15 images matched with each class. The reconstruction was made by back projection based on the 52 class means. The threshold used (see Figure 4d) was chosen to include the correct molecular weight. The resolution was determined to be 2.1 nm by Fourier shell correlation (50% value).

## Results

### Purification of the *H. marismortui* 20S proteasome

We identified two bands on SDS-PAGE, with  $M_r$  of 32 and 25 kDa, that co-purified with proteasome-containing fractions. These proteins were purified to homogeneity by a protocol in which all steps were performed in high salt concentration to maintain halophilic protein stability. An SDS-PAGE gel is shown in Figure 1. In the first steps the "salting out" effect of the high ammonium sulfate concentration stabilized the proteins, which were loaded on a Sepharose 4B column used as a hydrophobic interaction column. Bound proteins were eluted with a negative ammonium sulfate gradient. Fractions containing the proteasome were pooled and the ammonium sulfate concentration was increased to allow binding of the proteins on an anion-exchange column. A negative ammonium sulfate gradient combined with a positive sodium chloride gradient was used for protein separation. This step had a poor yield but was necessary to obtain good recovery from the subsequent hydroxyapatite affinity chromatography step. The 20S proteasome was finally separated from other contaminat-

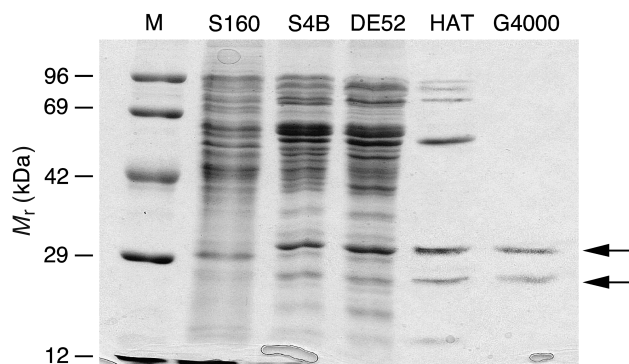


Figure 1. SDS-polyacrylamide gel electrophoresis of fractions from different purification steps of the proteasome from *H. marismortui*. Abbreviations: M, molecular weight markers; S160, *H. marismortui* extract after ultracentrifugation; S4B, proteasome pooled after Sepharose 4B chromatography; DE52, proteasome pooled after DEAE-Sepharose chromatography; HAT proteasome pooled after hydroxyapatite chromatography; G4000, pure proteasome pooled after gel filtration on a G4000 HPLC column (TosoHaas). The arrows indicate the  $\alpha$  and  $\beta$  subunits of the proteasome.

ing high molecular weight complexes by gel filtration in hyper-saline conditions using a G4000 column mounted on an HPLC system. After calibration of the column with non-halophilic proteins,  $M_r$  of the native particle was estimated to be about 700 kDa. Electron microscopy analysis allowed unambiguous identification of the complex as the 20S proteasome (see below). The purified particle consisted of two subunits of 32 and 25 kDa corresponding to the  $\alpha$  and  $\beta$  subunits, respectively.

### Peptidase activity and stability of the *H. marismortui* proteasome in different salt concentrations

Archaeal proteasomes exhibit high activity with chymotryptic substrates. The fluorogenic peptide Suc-Leu-Leu-Val-Tyr-AMC was chosen to test for endopeptidase activity. Potassium chloride was chosen as the standard salt instead of NaCl because KCl accumulates in all living cells. We found that, like the *H. volcanii* proteasome, the optimum temperature for Hm proteasome endopeptidase activity was 60 °C when measured in an assay buffer containing 2 M KCl (data not shown). High salt adaptation often leads to an increase in protein stability over a wide range of environmental conditions (Zaccai et al. 1989, Madern et al. 2000). This adaptation could be the result of an increased number of salt bridge clusters, as has been observed in HmMalDH (Richard et al. 2000). At 40 °C, the purified proteasome complex had a maximum specific activity of 2015 nmol min<sup>-1</sup> mg<sup>-1</sup> in 0.5 M KCl (Figure 2A). As with most of the halophilic enzymes studied thus far, maximum proteasome activity occurred at a salt concentration where the substrate protein was unstable (see below) (Mevarech et al. 1977). The peptidase activity of the halophilic proteasome showed a rapid drop in activity below 0.5 M salt. This is a common feature of halophilic enzymes and is believed to reflect denaturation of the enzyme, rather than a salt requirement for catalytic activity (Zaccai et al. 1989). The activity was three times lower in hypersaline salt solution, which is similar to the halophilic enzymes of the *H. marismortui* cytosol (Ginzburg et al. 1970). A decrease in enzymatic activity in high salt concentration has also been observed in other halophilic proteins (reviewed by Madern et al. 2000).

The Hm proteasome requires salt concentrations above 0.5 M KCl for stability (Figure 2B). This finding contrasts with most halophilic enzymes, including HmMalDH, which are unstable after long periods in salt concentrations below 2.5 M (Figure 2B). We conclude that the Hm proteasome displays high stability under salt conditions where halophilic proteins denature.

### Degradation of halophilic malate dehydrogenase by Hm proteasome activity

In vitro, the common substrates used to assay proteolytic activity of the 20S proteasomes consist of small proteins like casein or proteins that have been denatured by extreme thermal or oxidative stress (Rivett et al. 1994). Because Hm proteasome is stable in salt conditions whereas HmMalDH denatures, it was possible to study the proteolytic activity of the Hm



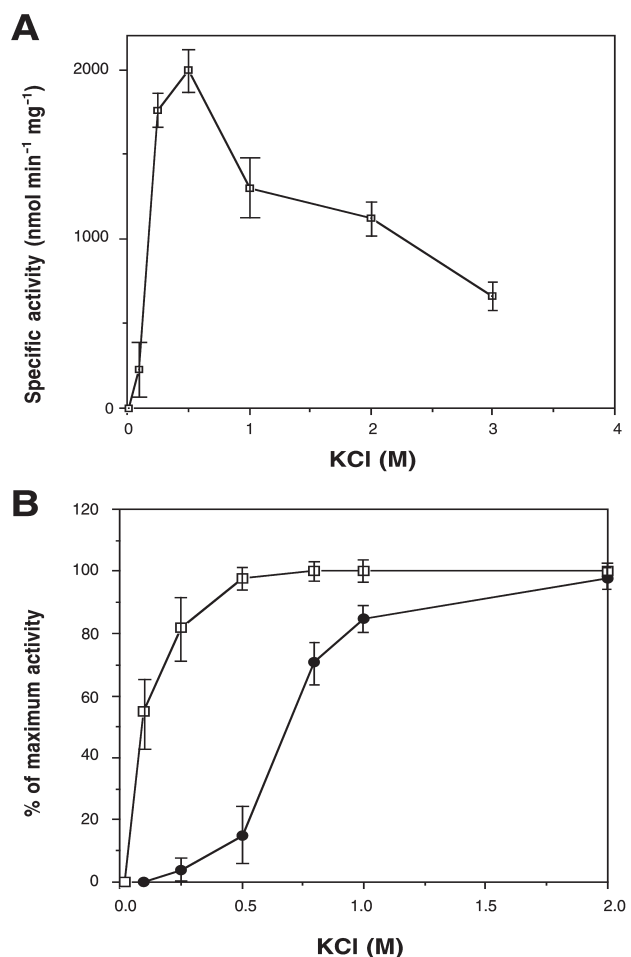


Figure 2. Salt-dependent activity and stability of the *H. marismortui* proteasome. (A) Halophilic proteasome activity. Peptidase activity was assayed by the hydrolysis of the synthetic peptide Suc-Leu-Lu-Val-Tyr-AMC. Continuous rate assays were performed in a thermostatically controlled fluorimeter and the initial reaction rate was measured at 40 °C in different KCl concentrations. (B) Effect of salt on the stability of the *H. marismortui* proteasome. Purified Hm proteasome (□) or Hm MalDH were diluted in various salt concentrations and incubated for 24 h (●). The residual peptidase activity was measured as in (A) in a reaction mixture containing 0.5 M KCl. The residual malate dehydrogenase activity was measured as described in Mader et al. (1995). Standard error bars are derived from three or four measurements.

20S proteasome during ongoing denaturation of the substrate protein. Peptidase activity assays were performed to ensure that the proteasome remained stable during incubation. We found that 0.4 M KCl is the limiting salt concentration below which the Hm proteasome begins to deactivate during incubation at 40 °C. Because we aimed to assess the proteolytic activity in different salt concentrations on folded and unfolded substrates, we first studied the denaturation rates of HmMalDH by fluorescence measurements and activity tests. The HmMalDH deactivated completely during a 1-h incubation in salt concentrations below 2 M. We verified that HmMalDH denatured by low salt concentration remained un-

folded even when returned to high salt concentrations such as 2 M KCl. No aggregation signal was observed during light scattering measurements indicating that, under our experimental conditions, HmMalDH denatured by low salt concentration is still soluble and therefore susceptible to proteolytic attack (data not shown). The same conclusion was drawn from previous neutron scattering, laser scattering and analytical ultracentrifugation experiments (Pundak et al. 1981, Zaccai et al. 1986).

The native or salt-denatured HmMalDH was incubated with proteasome in the assay mixture for 1 h. The data presented in Figure 3 demonstrate proteolysis of HmMalDH by the halophilic proteasome. The decrease in the specific HmMalDH signal on Western blots was used to assay for protease activity. Significant protease activity occurred in the presence of 0.4 M KCl when denatured HmMalDH was used as the substrate. Proteolytic activity was negligible at salt concentrations above 0.6 M (even over long periods). However, when native HmMalDH was used as a substrate (i.e., when the denaturation process occurred during the incubation with the proteasome) we found that the protein was degraded in all salt concentrations in which it destabilized (from 0.4 to 1.5 M KCl) (Figure 3). Note that the absence of HmMalDH degradation in salt concentrations above 2 M cannot be attributed to precipitation or denaturation caused by high salt concentration because studies show that HmMalDH is maximally soluble at KCl concentrations above 3 M (Ebel et al. 1999). The rate of cleavage was proportional to the rate of HmMalDH denaturation as estimated by deactivation kinetics. Therefore, we conclude that HmMalDH undergoing denaturation is a better substrate for the Hm proteasome than the fully denatured polypeptide chain.

### Three-dimensional structure of the halophilic proteasome

To determine possible differences between the structure of the halophilic proteasome and that of the thermophilic proteasome, we studied its 3-D structure by electron microscopy. Cryo-electron microscopy of proteins at high salt concentration does not provide reasonable contrast between the proteins and the aqueous medium. Therefore, we studied the proteasome with negative staining. Several stains were tested, and 1% ammonium molybdate gave the best results. The medium resolution results that were obtained from the 3-D reconstruction suggest that there was little or no particle flattening during the staining and air-drying procedure. The variance maps of the class mean did not show size differences. The field in Figure 4 shows end and side views. Often the particles in side view are attached at one or both ends to particles in end view, sometimes leading to the formation of chains. This tendency of the proteasome to interact end to side was not a problem for the reconstruction because the end-orientated proteasome never occupied exactly the same position on the side-orientated proteasome. During the alignment, all end views were averaged and we did not observe enhanced image density on completion of proteasome reconstruction (at the threshold image density giving the right volume according to the molecular

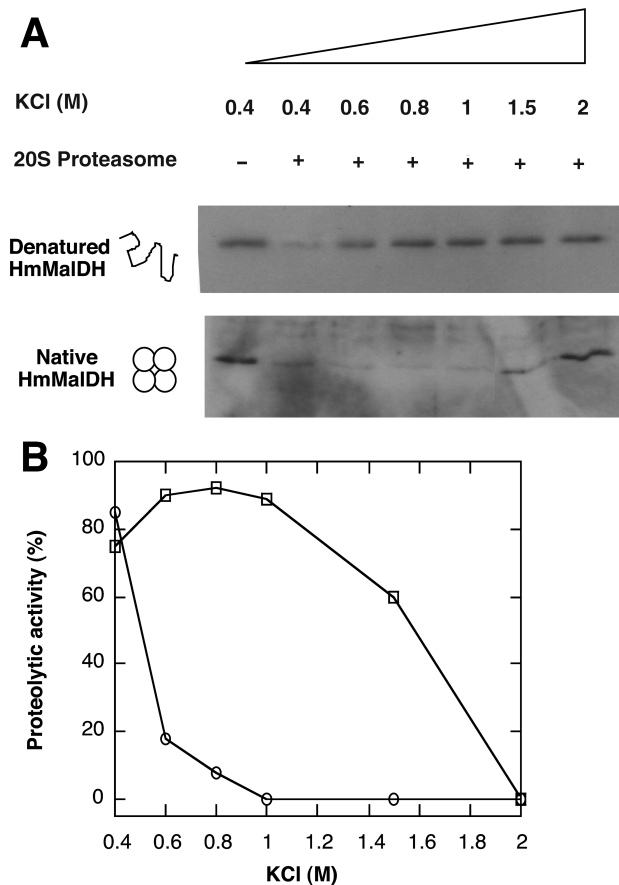


Figure 3. Halophilic proteasome processing of halophilic malate dehydrogenase (HmMalDH). The HmMalDH (native or denatured) was incubated at 40 °C with Hm proteasome at the indicated salt concentrations. (A) Protein breakdown was assessed by Western blot of the remaining HmMalDH. (B) Proteolytic activity with denatured (○) or native (□) HmMalDH as substrate as a function of KCl concentration. Western blot bands were scanned, and proteolytic activity was calculated as band intensity as a percent of control.

weight of the particle). The 3-D electron microscopy map clearly shows that the proteasome is made up of 14 monomers of each of two different protein subunits. The more spherical subunits are situated at both ends of the structure and the more elliptic subunits are located in the middle. Comparison between the electron microscopy map of the halophilic proteasome and the X-ray structure of the thermophilic proteasome from *Thermoplasma acidophilum* filtered to 2.0 nm shows that the two structures are similar in shape, as well as diameter and height ( $11 \pm 0.6$  and  $14 \pm 0.6$  nm, respectively). The halophilic proteasome, which functions in hypersaline conditions, and the thermophilic proteasome designed to function at temperatures close to 70 °C, have the same global structure, suggesting that no ultrastructural changes are necessary to adapt to different extreme environments. This also confirms that the proteasome is widespread and highly structurally conserved among archaea.

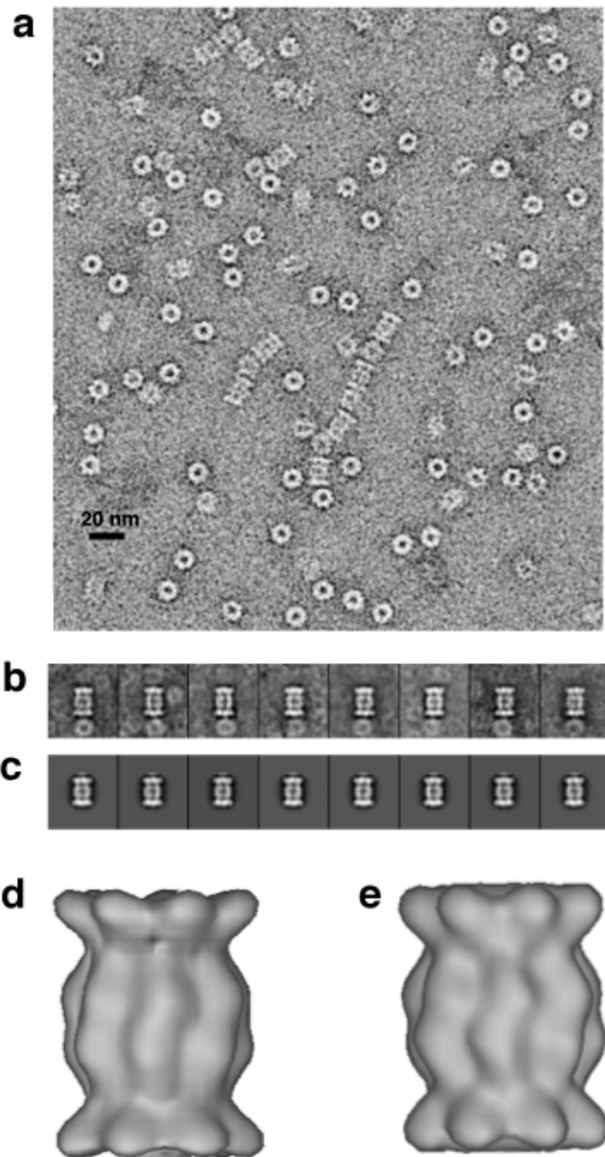


Figure 4. Negative staining and 3-D reconstruction of the halophilic proteasome. (a) Electron microscopy of a negatively stained sample of the halophilic proteasome. The image shows end and side views of the same object. The scale bar represents 20 nm. (b) Class means of the proteasome. Every class is the mean of 10–15 images. The side views are represented every 7° in the asymmetric unit. The rings below the side views are present because each side view sticks to an end view (see a). (c) Reprojected views of the proteasome. The side views are represented every 7° in the asymmetric unit. (d) 3-D reconstruction of the halophilic proteasome. (e) X-ray structure of the thermophilic proteasome filtered to 2.0 nm.

## Discussion

We have described the biochemical characterization of a proteasome from an extreme halophile, *Haloarcula marismortui*. An important contribution to the forces that stabilize a halophilic protein is the network of hydrated salt ions that cover the protein surface (Zaccai et al. 1989, Ebel et al. 1999). Consequently, halophilic proteins denature when the salt con-

centration decreases. We found that the halophilic proteasome in *H. marismortui*, like that in *H. volcanii*, requires a high salt concentration (over 1 M KCl) to remain stable, oligomeric and active over long periods; nevertheless, this typical halophilic feature is strikingly less pronounced than for other halophilic soluble enzymes like the tetrameric enzyme HmMalDH. Even at 0.5 M KCl, the rate of inactivation of the proteasome is considerably slower than that of HmMalDH (not shown). The Hm proteasome is also stable at salt concentrations that are far below the growth optimum for *H. marismortui*, suggesting that the enzyme is stable even when cells are exposed to hyposaline stress. The high stability of the Hm proteasome under conditions that normally denature halophilic enzymes prompted us to test its proteolytic activity in salt shock conditions. We showed that, in vitro, the 20S Hm proteasome ensures efficient breakdown of a halophilic protein under hyposaline conditions (below 2 M KCl). Taken together, these results suggest that the Hm proteasome functions to overcome the accumulation of low-salt damaged proteins in the cytoplasm and thus is part of a low-salt stress response system by which halophilic cells resist environmental changes. Other studies of archaea have also provided evidence that the proteasome functions to combat stress. For example, in thermophilic archaea, it was found that inhibition of proteasome activity reduces the ability of cells to tolerate heat shock (Maupin-Furlow et al. 1998, Ruepp et al. 1998).

We found that, when incubated with the Hm proteasome, fully denatured HmMalDH is degraded only in 0.4 M KCl, whereas native HmMalDH is processed in destabilizing salt conditions ranging from 0.4 to 1.5 M KCl. Electron microscopy of the Hm proteasome revealed that openings formed by the  $\alpha$ -subunits are too narrow to allow the entry of polypeptides with limited secondary structure to the central catalytic cavity. Therefore, as for other 20S proteasomes, we would expect that in vitro protease activity is restricted to a few small proteins that were previously fully denatured by heat, oxidative damage or disulfide bond reduction. On the contrary, the efficient breakdown of HmMalDH during its denaturation process suggests that partially folded proteins may constitute better substrates for the proteasome than fully denatured polypeptide chains. As observed for molecular chaperones, this could be associated with specific recognition of partially folded proteins (folding intermediates) through the transient exposure of hydrophobic patches (Badcoe et al. 1991, Martin et al. 1991, Ellis 2000), which could explain why HmMalDH is better degraded during denaturation. A similar recognition mechanism that involves increased surface hydrophobicity has also been proposed to explain why oxidized soluble proteins are efficiently degraded by the eukaryotic 20S proteasome in a ubiquitin-independent manner (Pacifi et al. 1993).

Eukaryotic cells contain a ubiquitin-tagging system that targets the folded proteins to the proteasome via regulatory multi-subunit complexes such as the 19S (PA 700) complex, which activates protein breakdown by the 20S proteasome (Ciechanover 1998). Ubiquitin or ubiquitin-processing genes

have not been found in any of the completely sequenced genomes of archaea, but PAN, a homolog of the ATPases from the eukaryotic 19S regulatory complex (Rpts), has been reported in the archaeon *Methanococcus jannaschii* (Zwickl et al. 1999). This complex has been shown to activate the in vitro degradation of denatured casein and folded proteins (Zwickl et al. 1999, Benaroudj and Goldberg 2000). Two genes were identified as PAN homologs in the genome from *Halobacterium* sp., an extreme halophile (Ng et al. 2000). The breakdown of abnormal cellular proteins is ATP dependent in vivo, suggesting that the 20S proteasome cannot function independently of PAN. Our data suggest that the contribution of such a complex is not a strict requirement for the functioning of the Hm proteasome in extreme salt shock conditions. However, it is likely that the crowded environment of the cell cytosol would influence the accessibility of substrate proteins to the active site and that, in these conditions, PAN may be required. Also, the 3-D structure of the Hm proteasome obtained from image reconstruction shows no structural difference compared to the 20S proteasome from *T. acidophilum*, which could account for its autonomous functioning of Hm proteasome. In conclusion, this in vitro study shows that the 20S halophilic proteasome is stable and functional in hyposaline conditions. It can efficiently cleave a halophilic protein during its denaturation by low salt concentration without the assistance of ATP-dependent regulatory complexes. The relevance of this finding for the in vivo functioning of the archaeal proteasome needs further exploration.

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